Inducing Age Related Changes in Microglia as a Model for Neurodegenerative Disease

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Inducing Age Related Changes in Microglia as a Model for Neurodegenerative Disease

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Abstract

Neurodegenerative diseases like Alzheimer's Disease and Parkinson's Disease are one of the biggest issues in healthcare that society is facing worldwide. Aging is a major risk factor for many neurodegenerative diseases and is thought to play a large role in their pathology. It is thought that the brain's resident immune cells microglia can change with age to obtain a detrimental phenotype that disrupts tissue homeostasis and promotes conditions for neurodegenerative disease to develop. Therefore, understanding and implementing microglial aging into the study of neurodegeneration could be important to further advances in the area.

A model of aging microglia is presented in this thesis. One of the marked characteristics of aging microglia is their accumulation of iron. Supplementing microglia with excess iron caused them to take it up and change into an iron-fed phenotype that resembles aged microglia. This was demonstrated by disrupted proteostasis and an altered secretory phenotype including the release of inflammatory cytokines and reactive oxygen species.

This model was applied to the study of Alzheimer's disease and it was found that the molecules secreted by iron-fed microglia degraded β-amyloid secreted by a neuronal cell line at a lower rate. The reduced degradation was due to the iron-fed microglia releasing lower levels of the protease insulin-degrading enzyme (IDE). Further study showed this decreased release of IDE was a result of disrupted autophagic flux caused by increased endoplasmic reticulum stress.

It was also shown that the aged microglia model can be applied to the study of Parkinson's disease. Increased levels of tumour necrosis factor-α secreted by iron-fed microglia was found to induce an increase in α-synuclein expression. Increased α-synuclein expression was found to sensitise neuronal cells to the toxicity of α-synuclein oligomers. It was found that this effect was conferred through an increase in Forkhead box O 3a expression that was caused by elevated iron levels due to the increased ferrireductase activity of α-synuclein.

The findings of these studies show that changes that occur in microglial aging are capable of increasing the deposition of proteins thought to be central to the pathology of the most common neurodegenerative diseases. These results suggest that iron-fed microglia are a promising model of microglial aging and have great potential utility to be applied to the study of both neurodegenerative disease and aging itself.

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1. Introduction

1.1 General introduction

Neurodegeneration is most commonly represented by diseases such as Alzheimer's Disease and Parkinson's Disease. Such diseases are becoming an increasingly common and pressing issue worldwide. After many decades of research, some progress has been made in understanding these deadly conditions, but we are still far from completely unravelling the complex processes involved in their molecular pathology. As of now, most neurodegenerative diseases are incurable, and it's not known how to prevent them. Accumulation of aggregated proteins has been shown to play a central role in the pathology of Alzheimer's disease (β-amyloid) and Parkinson's disease (α-synuclein).

Aging leads to a multitude of changes in the brain that in many ways are similar to those of neurodegeneration. In fact, the biggest risk factor for neurodegenerative diseases is aging and it is thus imperative to understand in what way it affects their pathogenesis. However, aging is rarely incorporated into the study of neurodegeneration, partly due to the fact that it is not a process that is completely understood, but also due to the heterogenous changes that occur in a multitude of molecular pathways.

Microglia are now thought to be central to the neuroinflammation seen in aging and age-related neurodegenerative disease. Release of inflammatory molecules and toxic substances by microglia is thought to be a major driver of neuronal death but what causes this increase in inflammation has not been agreed upon.

It has been shown that microglia change with age, but microglial aging is still poorly understood. It is linked both with increased release of inflammatory signaling factors such as cytokines and ROS but also reduced phagocytosis and motility. Modelling aging microglia in the brain would be helpful in not only understanding how neurodegenerative diseases occur to begin with, but also in gaining a better idea of what specific molecular environment in the brain triggers neuronal death in those diseases.

1.2 Neurodegenerative Diseases

Neurodegeneration is the progressive loss of function and death of neurons in the central nervous system (CNS) or the peripheral nervous system (PNS). It can be the result of hypoxia, trauma, stroke, poison, infections, cancer, diabetes but also due to chronic diseases that directly affect neurons known as neurodegenerative diseases (ND) (Przedborski et al. 2003). Depending on where and how the loss of neurons can occur, a variety ND has been identified. ND are incurable and typically result in years of progressively decreasing brain function and death. As ND are the most common reason for dementia, they result in an enormous physical, psychological, social and economic impact on society. Current numbers from the World Health Organisation (WHO) show that right now around 50 million people around the world live with dementia, with almost 10 million new cases being diagnosed every year (WHO 2017).

A myriad of ND has been identified with a few conditions being the most common. Alzheimer's Disease (AD) is by far the most common neurodegenerative condition with an incidence of 5-8% over the age of 65 years, climbing to 25-50% over the age of 85. (Duthey 2013; Przedborski et al. 2003). The second most common ND is Parkinson's Disease (PD) with an age-adjusted incidence of 9.7 to 13.8 per 100,000 population per year worldwide and up to almost 3% over the age of 80 (Hirsch et al. 2016; de Lau and Breteler 2006; WHO 2014). Less common but still important forms of neurodegeneration include Huntington's disease (HD), Amyotrophic Lateral Sclerosis (ALS), prion protein diseases and many others. ND can be the result of genetic disorders (AD, PD, HD, ALS), environmental exposure to toxic agents (possibly PD) or can be 'sporadic' with no clear cause (AD, PD, ALS).

1.2.1 Risk factors for developing neurodegenerative disease

The biggest risk factor for many major ND is age. With the increase in longevity worldwide seen in the last century the likelihood of an individual developing a ND in their lifetime has increased. Apart from age, other risk factors for developing sporadic neurodegeneration have been identified. Gender affects the incidence of the most common ND, where women are more likely to develop AD, but men have an increased risk of PD. Common health conditions such as obesity, diabetes and hypertension seem to increase the risk of developing cognitive impairment (Duthey 2013). There is also evidence that traumatic brain injury increases the risk of developing AD, PD and ALS (Louw et al. 2011). Lifestyle factors seem to also play a role in some ND symptoms. Some studies have linked an unhealthy diet, tobacco and alcohol use with increased risk of cognitive impairment (Mayeux and Stern 2012). Modifiable risk factors for dementia include depression, social isolation and cognitive inactivity (WHO 2017). Being aware of the risk factors for developing ND has not led to major advances in prevention or treatment, possibly due to significant gaps in understanding the underlying molecular cause of these conditions, with any treatments available being largely symptomatic.

The pathophysiology of ND is generally characterised by a central loss of neurons accompanied by reactive gliosis (Pekny and Pekna 2016). Another common link for many ND is the abnormal accumulation of misfolded, oligomerised or aggregated proteins in neurons and in the extracellular space and an increase in oxidative stress (Zhao and Zhao 2013; Kim et al. 2015). Aggregated proteins are thought to be central to disease pathology

and thus AD, PD, HD, prion diseases and others are termed proteopathies (Walker and LeVine 2000).

Death of neurons in ND could be caused by the triggering of endogenous programmed cell death pathways. Cell death in AD and PD is thought to occur through apoptosis, necrosis and necroptosis, but identifying the dominating mechanism has proven to be difficult (Jellinger 2001; Caccamo et al. 2017; Zhang et al. 2017). Apoptosis is generally defined as cell death that comprises of cellular shrinkage, nuclear fragmentation and chromatin condensation that culminate in membrane fragmentation with unaffected organelle integrity. Many signals can trigger apoptosis in neurons. Absence of neurotrophic factors, excess of glutamate, oxidative stress and environmental toxins are all factors that can cause neuronal death (Kermer et al. 2004). Proteins thought to initiate the apoptotic process in neurons include caspases, Bcl-2-associated X protein (BAX), Bcl-2 associated death promoter (BAD), glutamate receptors, Fas cell surface death receptor (FAS), prostate apoptosis response protein 4 (PAR-4) and tumour protein 53 (p53) (Mattson 2000). Mitochondria are thought to be central to initiation of the cell death process through the production of reactive oxygen species. The presence of misfolded proteins such as those in AD and PD and reactive oxygen species can cause apoptosis in neurons through impairing mitochondrial activity and causing endoplasmic reticulum stress (Bredesen et al. 2006). Endoplasmic reticulum stress- induced apoptosis has been documented in neurodegenerative disease. Stress sensors that are activated by ER stress lead to increased activity of the transcription factor C/EBP homologous protein (CHOP) that upregulates pro-apoptotic pathways. The activation of proteins such as bcl-2 interacting mediator of cell death (BIM) or p53 upregulated modulator of apoptosis (PUMA) is required for this type of apoptosis. CHOP is thought to interact with Forkhead Box O3a (FOXO3a), a member the FOXO transcription factor family in order to activate these proteins and trigger apoptosis in neurons (Ghosh et al. 2012; Essafi et al. 2005; Sanphui and Biswas 2013; Puthalakath et al. 2007). A newly identified form of programmed cell death – necroptosis has been shown to be implicated in AD and PD (Caccamo et al. 2017; Iannielli et al. 2018). It is thought to be initiated by receptor-interactive protein kinases (RIP) 1 and 3 and mediated by mixed lineage kinase domain-like (MLKL) protein. It is thought to be initiated as a response to tumour necrosis factor α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL) and interferons (IFN) among others (Zhang et al. 2017). Investigations into the role of necroptosis in ND are suggesting that it could be a powerful therapeutic target but they are still in their early stages.

1.2.2 The role of FOXO3a in apoptosis and neurodegenerative disease

Forkhead Box O3 (FOXO3a) is a transcription factor that activates many pathways that impact cellular viability. FOXO3a is evolutionarily conserved from simple species such as nematodes or the hydra, through *Drosophila*, to mice and humans, indicating that the pathways controlled by FOXO3a and its homologues are essential to survival (Martins et al. 2016; Anton-erxleben et al. 2013). The first forkhead box transcription factor found to have an effect on lifespan was the gene *daf-16* expressed in *C.elegans* (Kenyon et al. 1993; Dorman et al. 1995). It was also the first to be linked to the insulin/IGF-like signalling pathway, suggesting that nutrient sensing mechanisms have an effect on lifespan (Partridge and Brüning 2008). The *Drosophila* ortholog of *daf-16* - dFOXO has also been linked with lifespan extension mediated by altered nutrient sensing (Hwangbo et al. 2004; Giannakou et al. 2004). These findings have increased the interest in forkhead

box genes in mammalian aging research. While more primitive organisms have only one FOXO protein, mammals have 4 which are expressed in almost all tissues. FOXO3a is quite ubiquitous and is highly expressed in the brain and is therefore to particular interest to the study of age-related neurodegenerative disorders (Klionsky et al. 2009). The structure of FOXO3a is similar to that of other forkhead box proteins (Figure 1.1). It has a DNA binding domain (DBD or the forkhead box) followed by a nuclear localization signal, a nuclear export sequence and a transactivation domain (TAD) (Calnan and Brunet 2008).

Variants of FOXO3a have been found to be linked to longevity in humans. Studies of very old individuals in various populations (Germans, Danish, Han Chinese, Americans, Italians) have linked single nucleotide polymorphisms (SNPs) in FOXO3a with increased lifespan (Zeng et al. 2010; Martins et al. 2016). These findings have resulted in substantial interest in the mechanism through which FOXO3a could confer these beneficial effects. The SNPs identified were intronic so did not belong to the coding regions of the gene. Therefore, it is possible that they affected the amounts of FOXO3a protein in the patient's tissues instead.

FOXO3a activity can be stimulated by oxidative stress or caloric restriction. The IGF-1 signalling pathway is the best studied mechanism of FOXO3a regulation (Figure 1.1) (Brunet and Webb 2015). It is also a widely evolutionarily conserved mechanism that affects rates of aging. Insulin or IGF-1 binds to an insulin or IGF receptor which eventually results in the activation of AKT kinase. AKT phosphorylates FOXO3a and that results in its nuclear export and therefore its inhibition. Loss of function mutations in this pathway have been linked to increased human longevity. FOXO3a has been found to interact with different proteins apart from AKT kinase. FOXO3a has also been found to be phosphorylated by SGK, IKKβ, and ERK among others (Calnan and Brunet 2008). FOXO3a activity can also be modulated by acetylation and de-acetylation (Daitoku et al. 2011). Acetylation occurs by enzymes such as p300 and is a modification that can have an activating or inhibitory effect.

Another protein implicated in aging (SIRT1) has been found to de-acetylate FOXO3a (Wang et al. 2012; Giannakou and Partridge 2004; Hori et al. 2013). The effect of SIRT1 deacetylation seemed to promote the cell-cycle arrest and oxidative stress alleviation activities of FOXO3a but inhibit the pro-apoptotic action of the protein and possibly target it for degradation by ubiquitination. On the other hand, AMP-activated protein kinase (AMPK) has been found to increase FOXO3a activation and promote its proapoptotic activity (Li et al. 2017). It seems that the role of the many post-translational modifications FOXO3a undergoes is not just overall modulation of activity, but also promotion of particular FOXO target pathways that can have various consequences on viability and longevity.

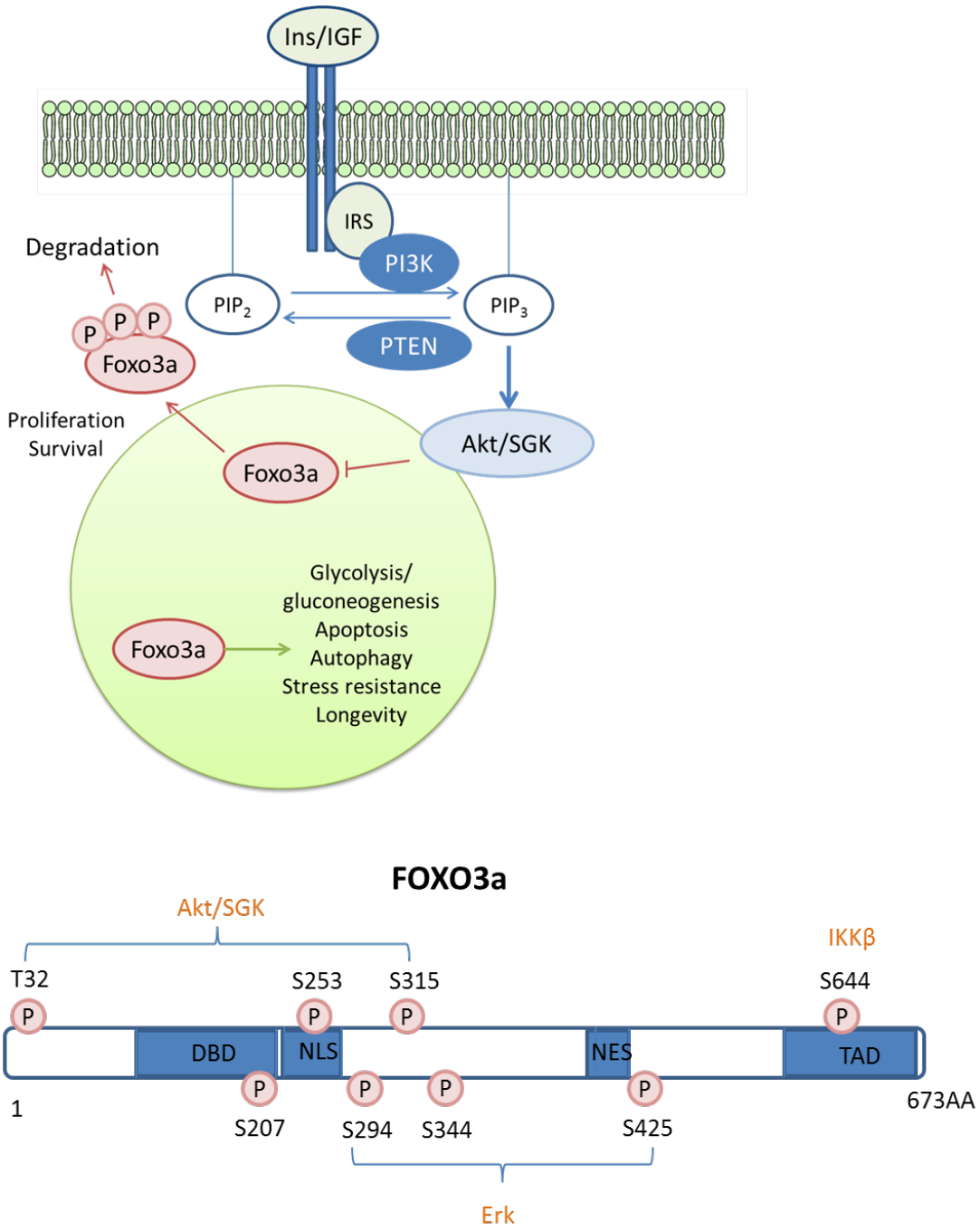

Figure 1.1 Simplified FOXO3a pathway and FOXO3a structure. This figure displays the IGF/Insulin pathway that results in the inactivation and trafficking of FOXO3a from the nucleus through AKT or SGK phosphorylation. The structure of FOXO3a schematic includes the main domains of the FOXO3a protein and the phosphorylation sites for AKT/SGK, ERK and IKKβ.

FOXO3a activity could play a role in longevity in multiple ways. Firstly, it works to stimulate autophagy. This could occur through the direct upregulation of autophagy through the expression of inducers such as BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) (Mammucari et al. 2007). Additionally it has been shown to promote autophagy through a FOXO1 dependent mechanism where FOXO3a activity results in increased FOXO1 phosphorylation which can bind Autophagy-related protein 7 (Atg7) and promote autophagy (Zhao et al. 2010; Zhou et al. 2012). Loss of protein homeostasis is a hallmark of age-related decline in organisms and is implicated in a myriad of

neurodegenerative disease as previously mentioned. FOXO3a activity is also linked to increased proteasomal degradation of cytosolic proteins through increased expression of ubiquitin ligases and proteasome components, another mechanism that is impaired in the aging organism. FOXO3a is also implicated in resistance to oxidative stress. Some of its main targets are MnSOD, catalase and GADD45, enzymes which neutralise ROS. Appropriate balance in the levels of ROS in the cell is very important for viability since they are used as signalling molecules but can damage cellular structures if in excess. ROS damage and elevated oxidative stress has been heavily implicated in neurodegenerative disease, to the point where antioxidant therapies have been trialled for treatment of AD and PD (Kim et al. 2015). Additionally, FOXO3a deletion has been found to disrupt the function of stem cells, which are integral to the regeneration of aging organisms. FOXO3a is also considered to be a type of tumour suppressor protein as it can trigger genes such as *BIM*, *TRAIL* and *PUMA* that code for pro-apoptotic proteins. Conversely, excessive and ill-timed FOXO3a activation can lead to the inappropriate growth arrest and apoptosis of healthy tissues. In fact FOXO3a inhibition has been shown to be neuroprotective against oxidative stress (Chong et al. 2004)

As FOXO3a is implicated in so many mechanisms related to cell viability it's not surprising that it also plays a role in neurodegenerative disease. Levels of FOXO3a have been found to positively correlate with cognitive impairment and β-amyloid load in AD (Sahin et al. 2013). High levels of FOXO3a have been measured in the cortical neurons of aged and AD brains (Sahin et al. 2013). β-amyloid levels were also found to correlate with FOXO3a levels in mouse neurons (Qin et al. 2009). In a recent study it was found that the increased FOXO3a levels are a response to APP processing by γ-secretase, likely through action of the APP intracellular domain reducing phosphorylation of AKT (Law et al. 2017). Pink1 is a pro-survival gene, loss of function of which is implicated in the pathology of PD. It is also a target for FOXO3a, which induces its transcription resulting in cellular survival (Mei et al. 2009). Interestingly, FOXO3a has been found to be ectopically expressed in Lewy bodies. FOXO3a has also been found to be induced by increases in α-synuclein, probably due to its effect on autophagy. A low-level increase in FOXO3a activity can be neuroprotective by initiating the degradation of the α-synuclein aggregates. However, constitutive activation of FOXO3a resulted in the death of dopaminergic neurons (Pino et al. 2014; Law et al. 2017).

It is evident that FOXO3a is a protein implicated in many cellular processes and its regulation is multifaceted and intricate. The timing and type of activation of FOXO3a can contribute to either neuroprotection and increased longevity or neuronal apoptosis and aging (Maiese 2017).

1.3 Alzheimer's Disease

Alois Alzheimer's investigation published in 1907 described and named the main hallmarks of the AD brain (Hippius and Neundörfer 2003). AD accounts for the majority of cases of dementia in the world (Masters et al. 2015). More than 95% of patients suffer from the sporadic form of AD that is characterised by a late onset at 80-90 years of age (Masters et al. 2015). Less than 1% of patients suffer from a heritable form of AD with a mean age of onset of 45 years. Those cases of AD are directly caused by mutations in genes that code for proteins that contribute to the formation of amyloid plaques (APP, PSEN1, PSEN2). However, the genetic component of the vast majority of cases acts as a risk factor. The most significant example of this is the ε4 isoform of apolipoprotein E (APOE4) which is expressed in more than half of caucasian AD patients and can increase risk of developing AD up to 12-fold (Michaelson 2014). APOE has been found to interact with β-amyloid and the isoform APOE4 is thought to contribute to the formation of plaques by chronically decreasing β-amyloid clearance (Kim et al. 2009). Variants of the protein triggering receptor expressed on myeloid cells 2 (TREM2) have also been linked to higher risks of developing AD. Partial loss of function mutations in TREM2 which is expressed on the cell membranes of microglia reduce their ability to react to and clear β-amyloid (Carmona et al. 2018).

AD is characterised by the loss of synapses and death of neurons in many brain regions including the hippocampus and the cerebral cortex. That results in progressive memory loss, changes in personality, loss of independence and eventually loss of basic body function, resulting in death. Symptoms of mild AD include forgetfulness, confusion, poor judgement, mood and personality changes which intensify as the disease progresses and problems with speech, language, disturbed sleep among others also arise. In the final stages of AD, the aforementioned symptoms become more severe with significant problems with short and long term memory continuing and symptoms such as dysphagia, incontinence, and loss of muscle tone developing as well (NHS 2018). AD is the most common cause of dementia worldwide, being the culprit for 60-70% of cases (WHO 2017). AD is a chronic disease with the clinical stage lasting about a decade, but currently it is thought to be preceded by a pre-clinical stage of around two decades. The neuronal death that occurs affects both cholinergic and dopaminergic neurons. However, synaptic dysfunction seems to precede neuronal death and is enough to result in cognitive impairment. Normally the pathology is first detected in the frontal and temporal lobe and spreads at varying rates to other cortical areas (Masters et al. 2015). AD is characterised as a proteopathy because one of its major hallmarks is the accumulation of proteins in amyloid plaques and neurofibrillary tangles which have been implicated in neuronal death (Walker and LeVine 2000).

Protein aggregation is a central event in AD. Amyloid plaques have been described as an accumulation of insoluble forms of the peptide β-amyloid. They are found in the extracellular space, but also in blood vessel walls. Plaque deposition has been found to precede the appearance of neurofibrillary tangles and to occur in the front and temporal lobes, hippocampus and the limbic system and to spread to other areas. Neurofibrillary tangles are aggregates of the hyperphosphorylated microtubule protein tau and are found in neurons. Tangles are initially found in the medial temporal lobes and hippocampus with the pathology spreading to other cortical areas with disease progression. The cause of

formation of tangles is unclear but β-amyloid is thought to play a role in increasing tau cleavage and hyperphosphorylation (Selkoe and Hardy 2016). A protease upregulated during aging called asparagine endopeptidase has been recently shown to cleave tau and enhance its aggregation (Zhang et al. 2014). The deposition of β-amyloid in AD is considered the disease-triggering event, however the mechanisms that result in this deposition and the exact role risk factors such as age play in it have not been fully unravelled (Allsop and Mayes 2014; Hardy and Allsop 1991). Protein aggregation in AD starts in the pre-clinical stages in the disease and thus those stages are of particular interest in terms of detection and treatment.

Clinical trials for AD treatments have as of now yielded limited results in terms of both prevention and neuronal rejuvenation. Current available medications only manage disease symptoms by modulating the signalling of neurotransmitters such as acetylcholine and glutamate. Cholinesterase inhibitors such as donepezil and galantamine enhance acetylcholine signalling by increasing the time it is present in the synapse. Cholinesterase inhibitors are commonly applied in mild AD. N-methyl-d-aspartate A (NMDA) receptor antagonists such as memantine normalise glutamate signalling. Memantine has been found to have benefits in moderate AD with mild improvements in cognitive ability. Combination therapy with both kinds of agents only results in a short delay in progression of symptoms of 6-9 months (Masters et al. 2015). A big issue in treating AD effectively is that neuronal death is irreversible so treating patients who are already showing symptoms may be too late. Advances have been made in AD detection, with methods to measure β-amyloid accumulation in pre-symptomatic patients improving (Roe et al. 2017). In-vivo positron emission tomography (PET) imaging of β-amyloid and tau accumulation in the brain by using dyes that specifically bind these proteins can now be achieved with great sensitivity (Masters et al. 2015). This has made the identification of patients in pre-symptomatic stages of AD possible. Additionally, it is now feasible to measure changes in cerebrospinal fluid (CSF) metabolites that can predict familial AD at least a decade before symptoms develop (Fagan et al. 2014). Reducing β-amyloid levels in patients via monoclonal antibodies or pharmacological inhibitors have recently been pursued with limited success (Gold 2017; Yanagisawa 2018). Preventing protein aggregation in the preclinical stages is probably the key to stopping Alzheimer’s disease progression (van Dyck 2018; Masters et al. 2015).

1.4 Parkinson's Disease

Parkinson's disease is a neurodegenerative disease caused by the loss of dopaminergic neurons in the *substantia nigra pars compacta* located in the midbrain. The neuronal degeneration and death results in a reduction in dopaminergic neurotransmission in the striatum. This results in loss of inhibitory signalling pathways altered activity of both the striatum and motor cortex. Symptoms arising from these changes include bradykinesia, tremors, gait changes and loss in muscle control. PD is a chronic disease that causes significant disability, reduced quality of life and life expectancy. It was first described by James Parkinson in 1817 in his work "An Essay on the Shaking Palsy". Non-motor symptoms are also very common in PD, one of the earliest being constipation and with most patients eventually developing dementia, hallucinosis, incontinence and postural hypotension.

PD can arise sporadically which covers the majority of cases with the underlying causes for the disease thought to be a combination between environmental factors and genetic predispositions. Hereditary PD occurs in a much smaller proportion of cases. Mutations in certain genes carry a significant risk for developing PD. Hereditary PD can be classified in three subsets (Poewe et al. 2017). Autosomal dominant PD can be caused by mutations in α-synuclein, leucine-rich repeat kinase (LRRK) and vacuolar protein sorting-associated protein 35 (VSP35). Early-onset PD can be caused by mutations in protein deglycase DJ-1, parkin, PTEN-induced kinase 1 (PINK-1), and DnaJ heat shock protein family (Hsp40) member C6 (DNAJC6). Complex genetic inheritance in PD can be caused by mutations in Cation-transporting ATPase 13A2 (ATP13A2), (gene product: phospholipase A2 group 6 (PLAG2G6), gene product F-box protein 7 (FBXO7), DNAJC6 and synaptojanin 1 (SYNJ1). Many other genes have been linked to PD but their direct relationship to the disease has not been identified. Inherited mutations in α-synuclein are of particular interest in PD because of the protein's central role in the pathology. Missense mutations in the α-synuclein gene have been shown to cause the classic PD phenotype while gene duplication or triplication cause early-onset PD with symptoms of dementia.

Neurodegeneration and especially PD have been linked to environmental exposure to toxins (Cannon and Greenamyre 2011). A well-known example of this is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a chemical that, when ingested, can cause the death of dopaminergic neurons in the *substantia nigra*, producing symptoms very much like those of PD. Exposure to other chemicals has been linked to increased risk of developing PD as well. The pesticides paraquat and rotenone in particular have been linked to PD by epidemiological studies and in animal models produce loss of dopaminergic neurons. Chronic exposure to paraquat has been shown to induce cellular oxidative stress. This in turn can trigger the mitochondrial intrinsic proapoptotic pathway mediated through the release of cytochrome C and the activation of caspase 9 (Franco et al. 2010). Paraquat has also been shown to induce ER stress and to cause DNA damage, both alternative pathways through which apoptosis could be triggered (Yang et al. 2009). Rotenone's mechanism of neurotoxicity is similar to that of paraquat and is driven by increased mitochondrial ROS that trigger cytochrome C and caspase-dependent apoptosis, but also by the induction of ER stress (Franco et al. 2010). Exposure to solvents has also been linked to increased risk of PD. Chronic trichloroethylene inhalation

has been shown to cause loss of dopaminergic neurons in rats and has been correlated with severity of PD symptoms.

Additionally, metal exposure has been of particular interest in the study of AD and PD. Exposure to aluminium, zinc, copper and lead has been linked to AD and aluminium and lead exposure in animal models has recapitulated some features of AD. In PD, iron and manganese exposure have been studied with manganese poisoning causing a syndrome similar in symptoms to PD. In ALS exposure to mercury and lead have been identified as risk factors. Increased iron levels in the *substantia nigra* have also been linked to younger age of onset of PD in men (Bartzokis et al. 2004).

Exposure to all these toxins is thought to cause oxidative stress and thus lead to neuronal loss. Environmental exposures have not been conclusively proven to be the underlying reason for all but a small minority of cases of neurodegeneration and the symptoms caused by these toxins only replicate a part of the pathology of common ND. However, what these risk factors illustrate is the sensitivity of the brain to oxidative stress caused by either chemical exposure or metal dyshomeostasis.

Diagnosis of PD still largely relies on the presence of motor symptoms. Currently available PD treatments only address management of symptoms by pharmacologically substituting the depleted dopamine with its' precursor L-DOPA. Dopamine replacement therapy has major motor side-effects and many non-motor symptoms are not affected by it. Deep brain stimulation of the subthalamic nucleus has also had some success in PD symptom management but has the same drawbacks as L-DOPA with the addition of requiring surgical placement of an electrode, making the treatment much riskier. No curative or preventative treatments have been identified yet and major strides need to be made with identifying robust biomarkers that allow for early pre-symptomatic diagnosis.

The second most defining characteristic of PD besides neuronal death is the accumulation of α-synuclein. Lewy bodies, the first identified protein aggregate in PD, are intracellular neuronal inclusions and are mainly formed of α-synuclein but also other proteins complexed with iron (Castellani et al. 2000; Spillantini et al. 1997). They begin to form in pre-motor symptomatic stages of the disease in the olfactory bulb, medulla oblongata and the dorsal pons. As PD progresses to motor symptom stages, Lewy bodies also appear in areas of neuronal death. The loss of neurons normally starts in the ventrolateral *substantia nigra* and later on spreads to dopaminergic neurons of other brain areas such as the midbrain, the basal forebrain and the neocortex. The molecular mechanisms behind neuronal loss in PD are thought to involve many factors such as α-synuclein toxicity, neuroinflammation, oxidative stress, mitochondrial dysfunction and perturbed autophagy; all of which interplay.

1.5 Proteins that play a role in neurodegeneration

Proteopathies are a class of diseases where one or more proteins develop structural abnormalities and become toxic to cells and tissues. The toxicity can be due to altered function, loss of function or aggregation (Walker and LeVine 2000). A common thread that links many of the ND is that they involve the accumulation and aggregation of proteins that can be toxic (Ross and Poirier 2004). As mentioned above, α-synuclein, β-amyloid and tau have been linked to the most common neurodegenerative diseases AD and PD, but also Dementia with Lewy Bodies, Frontotemporal Dementia, and Down Syndrome. Interestingly, other ND are also characterised by protein aggregation. A misfolded form of the prion protein (PrP) termed PrP^{Sc} is commonly found to aggregate in prion diseases. PrP^{Sc} accumulates extracellularly in a variety of forms that include plaques which can be seen in diseases such as Creutzfeldt–Jakob Disease (CJD), Kuru, Fatal Familial Insomnia, and Gerstmann–Straussler–Scheinker disease in humans and most famously in Bovine Spongiform Encephalopathy and scrapie in animals (Hughes and Halliday 2017). In Huntington's disease mutant huntingtin and ubiquitin aggregate in striatal neurons (Bates et al. 2015). In amyotrophic lateral sclerosis multiple ubiquitinated proteins aggregate in motor neurons (Hardiman et al. 2017). TDP-43 is another protein found in inclusions in a number of ND including AD and dementia with Lewy bodies (Davidson et al. 2011; Cook et al. 2008). What unites many of the aggregates found in ND is that they commonly share a β-sheet structure and that in their sporadic forms they are the result of a complex network of events that results in changes in the environment of the brain of which we have a limited understanding. Elucidating the underlying reasons for protein aggregation and toxicity in neurons is central to understanding these diseases. Considering that aging is a risk factor to the main neurodegenerative diseases, changes in the brain that occur with aging may contribute to the eventual loss of neurons in these disorders.

1.5.1 Amyloid precursor protein and ß-amyloid in neurodegeneration

1.5.1.1 Structure of APP

Amyloid precursor protein (APP) is a highly expressed protein in many tissues including not only the brain but also muscle, adipose tissue, the intestine and the skin (Puig and Combs 2013). The mammalian gene that codes for APP contains 18 exons and has multiple alternative splicing forms consisting of 695, 639, 714, 751 and 770 residues in length (Golde et al. 1990; Nalivaeva and Turner 2013; Tang et al. 2003). Of those alternative splicing forms, the 695AA one is most common in the nervous system (van der Kant and Goldstein 2015). APP is a member of a family of similar proteins like the amyloid precursor-like proteins (APLP1 and APLP2). APP, similarly to APLP1 and APLP2, is a transmembrane protein with a small N-terminal intracellular domain and a large C-terminal extracellular domain (Figure 1.2). Unlike APLP1 and 2 it also contains the β-amyloid domain. The extracellular domain of APP includes the E1 and E2 domains and part of the β-amyloid sequence that is also partially located in the cytoplasm. A Kunitz-type protease inhibitor domain can be found between the E1 and E2 domains, but it is not present in the 695AA form of APP. The E1 domain contains a heparin-binding/growth factor-like domain

(HBD/GFLD), and copper- and zinc- binding domains. The E2 domain includes another HBD/GFLD. The cytoplasmic domain of APP contains a YENPTY motif that is involved in protein-protein interaction.

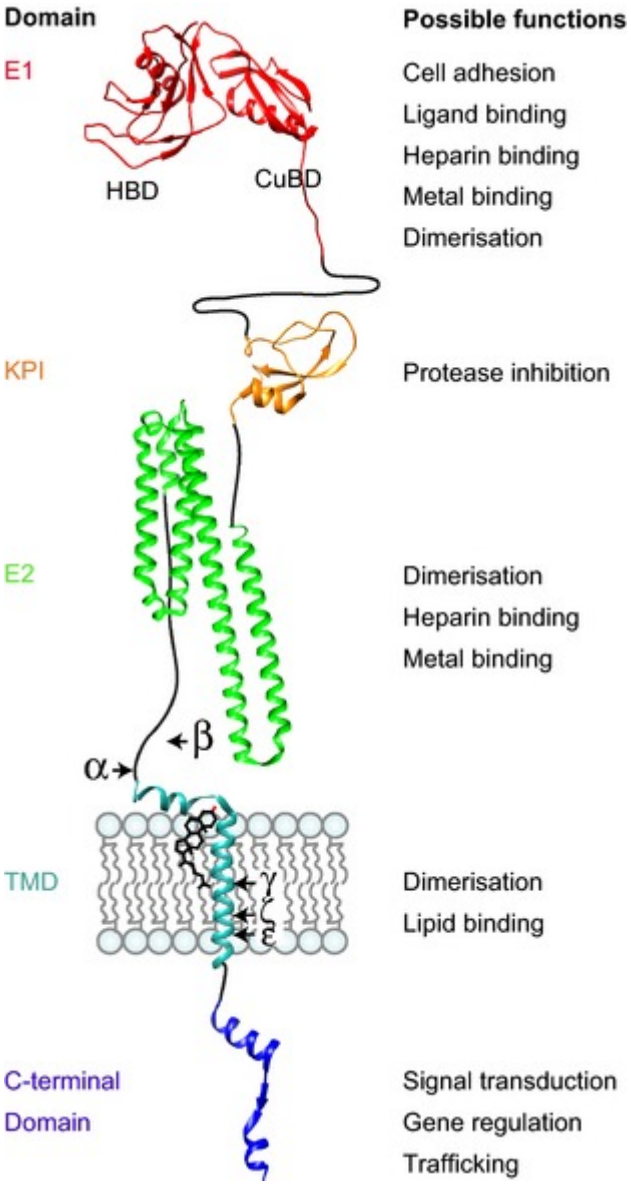

Figure 1.2 Hypothetical 3-dimensional structure of APP. Shown here is the proposed structure for APP which includes an E1 domain, Kunitz protease inhibitor (KPI) domain, E2 domain, transmembrane domain (TMD) and intracellular domain (C-terminal domain). The proposed lipid-binding site in the transmembrane domain is depicted with a cholesterol molecule bound to it. This structure includes the KPI domain which is not present in the 695AA isoform common in the brain. Taken from Dawkins and Small (2014).

1.5.1.2 Function of APP

The function of APP in the cell is still under dispute (Dawkins and Small 2014). It has been suggested to be heavily involved in the development of the nervous system, with possible roles in neuronal migration and synaptic formation and maintenance (van der Kant and Goldstein 2015). Putative functions of APP also include vesicle transport, maintenance of metal homeostasis, and regulation of intracellular calcium (Rogers et al. 2016; Müller and Zheng 2012). An emerging function of APP of some interest is the regulation of iron in the cell. Reductions in cellular iron inhibit APP translation through Iron-regulatory protein (IRP) binding an Iron Responsive element (IRE) found upstream of the coding region for APP (Cahill et al. 2009; Cho et al. 2010; Rogers et al. 2008). On the other hand, increases in iron also increase the levels of APP (Rogers et al. 2008). APP may play a role in iron efflux from the cells through the binding and stabilization of ferroportin (Wong et al. 2014). APP has been shown to be able to form dimers in the cell, a process that possibly occurs through its E2 domain (Lee et al. 2011). The dimerization process has been proposed to occur either between two APP molecules on the same cell membrane, intracellularly, or between APP molecules on two separate cells, suggesting a possible function of APP as a cell adhesion protein (Hoefgen et al. 2014; Soba et al. 2005). The E1 and E2 domains have also been shown to contain copper and zinc binding sites, with a possible iron binding site in E2 but this has been disputed (Wild et al. 2017; Honarmand Ebrahimi et al. 2013; Barnham et al. 2003). Interaction of APP with copper could affect both dimerization and rate of proteolytic degradation, possibly decreasing production of β-amyloid (Spoerri et al. 2012). APP is produced in the endoplasmic reticulum and transferred to the Trans-Golgi network. From there it is trafficked to the cell surface or an endosomal compartment via clathrin-associated vesicles where it undergoes proteolytic processing (O'Brien and Wong 2011). As APP is proteolytically processed into many peptides, it is possible that they are the ones that fulfil APP's function and indeed putative functions have been found for many of them (van der Kant and Goldstein 2015).

1.5.1.3 Proteolytic digestion of APP

APP can be cleaved by proteins called secretases, and one of those pathways generates β-amyloid (Figure 1.3). The enzymatic processing of APP can take two routes. The non-amyloidogenic pathway starts in the cell membrane where APP is cleaved by an α-secretase and then endocytosed and cleaved by a γ-secretase possibly in the trans Golgi network or in the late endosome. This process produces APP fragments that are not toxic, namely soluble APP α (sAPPα), C83, P3 and the APP intracellular domain (AICD). The amyloidogenic pathway occurs in endosomes where APP is cleaved by β-secretase and then γ-secretase enzymes. This generates the toxic β-amyloid fragment, but also sAPPβ, C99 and the AICD. The most likely candidate for α-secretase is considered to be the enzyme a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and for β-secretase - beta-site APP cleaving enzyme 1 (BACE1). γ-secretase is a protein complex that consists of four proteins: presenilin-1 or 2 (PSEN1,2), nicastrin, anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2). The soluble APP fragments sAPPα and sAPPβ seem to act as signalling molecules that increase neuronal

growth. sAPPα could also potentially act to protect neurons from excitotoxic stress. One suggested function of APP is a cell surface receptor similar to Notch. Netrin-1 and F-spondin have been identified as potential ligands for APP and their binding is thought to induce complex-forming with the protein FE65. Post-FE65 binding the APP intracellular domain AICD is thought to be translocated to the nucleus where it can act as a transcription factor to upregulate genes related to endoplasmic reticulum stress, β-amyloid degradation, and growth suppressor genes among others (Multhaup et al. 2015).

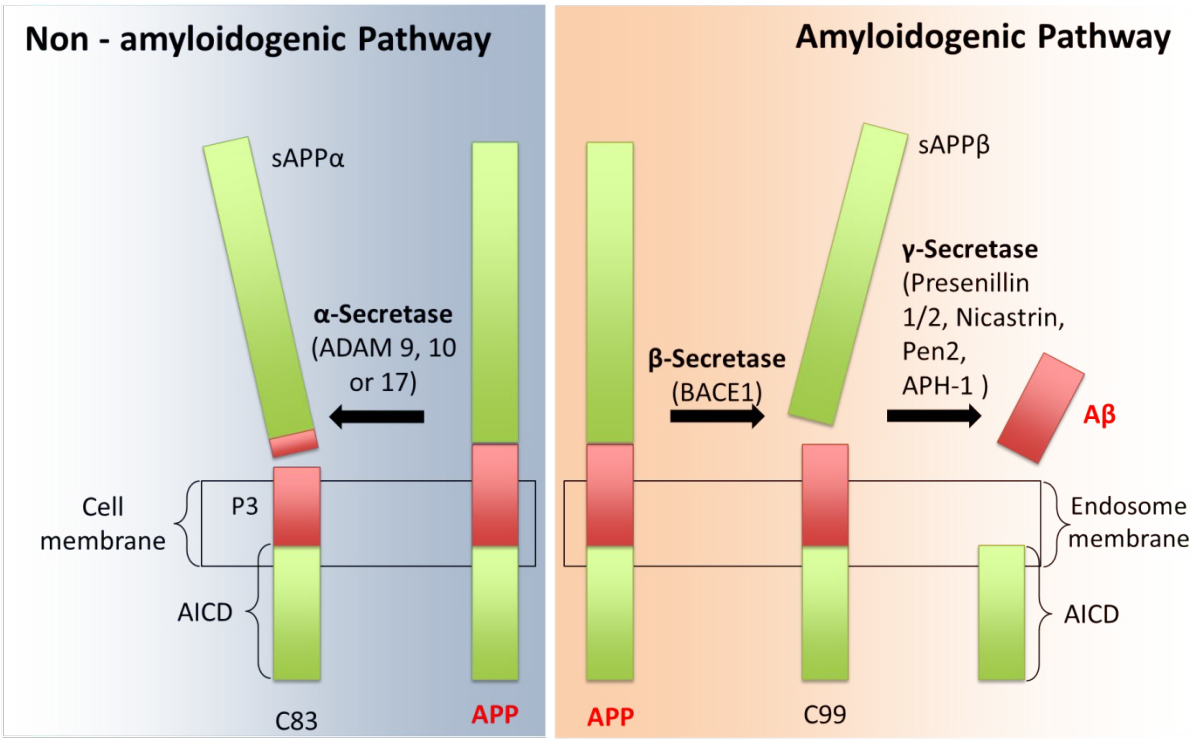

Figure 1.3 APP processing. This figure depicts the two main processing pathways for APP. The fragments APP is cleaved into by α, β and γ-secretase and the main candidates for those enzymes are also shown.

1.5.1.4 β-amyloid production and degradation

The peptide β-amyloid is the main component of amyloid plaques found in AD, which are a major pathological hallmark of the disease. It is thought that β-amyloid is a major contributing factor to neurodegeneration in AD (Hardy and Allsop 1991). This is not only due to the presence of amyloid plaques; but also due to the fact that some of the most common mutations linked to familial AD in the proteins PSEN1 and PSEN2 lead to an increase in the β-amyloid 40/ β-amyloid 42 ratio. Additionally, APP gene duplication in Down's syndrome leads to early β-amyloid deposition and onset of AD (Bird 1993).

Whether β-amyloid is just a by-product of APP processing or if it has a cellular function is currently unknown. It is possible that it can act as a signalling molecule. What is certain is that levels of β-amyloid in the brain are strictly regulated. In the healthy CNS β-amyloid has a short half-life of about 9 hours. The β-amyloid generated in neuronal late

endosomes can be degraded intracellularly or exported to the extracellular space. There it could also be degraded by proteases such as insulin degrading enzyme (IDE), neprilysin, matrix metalloproteases (MMPs) and others (Hernandez-Guillamon et al. 2015; Saido and Leissring 2012). β-amyloid can also be transported through the blood-brain barrier; and in fact, detection of β-amyloid in CSF and by positron emission tomography (PET) has become a commonly used biomarker for AD (Masters et al. 2015).

In the healthy CNS, β-amyloid is degraded intracellularly by the neurons themselves and extracellularly by glial cells, such as astrocytes and microglia, by the secretion of the β-amyloid degrading proteases mentioned above (Saido and Leissring 2012). Microglia and astrocytes have also been found to readily phagocytose β-amyloid both *in vitro* and *in vivo* (Lee and Landreth 2010). The accumulation of β-amyloid in AD brains can be explained not only by an increase in β-amyloid production but also a dysfunction in clearance especially as in the vast majority of AD cases there is no genetic component that leads to increased β-amyloid production. In fact, the biggest sporadic genetic risk factor for developing AD, APOE4 reduces the clearance of β-amyloid in the brain (Michaelson 2014). Additionally, studies have found that even in early stages of the disease there's evidence of reduced β-amyloid degradation with no detectable difference in production (Mawuenyega et al. 2010; Caccamo et al. 2005).

1.5.1.5 β-amyloid toxicity

Originally, it was thought that amyloid plaques were the cause of neurotoxicity in AD. That idea has come into question after many studies failed to correlate amyloid plaques with severity of dementia; as they are found a lot earlier than disease manifestation and their presence has been identified in post-mortem samples of many healthy individuals. Currently it is thought that soluble oligomeric forms of β-amyloid are the ones that confer the neurotoxic effect (Tabner et al. 2011). The sizes of β-amyloid produced in the brain vary, but the most common ones are the 1-40 and 1-42 amino acid isoforms. There is evidence that β-amyloid 1-42 is the more toxic of those two as it is more prone to oligomerisation and aggregation (Bitan et al. 2003; Qiu et al. 2015; Murphy and Levine 2010). Increases in the ratio of β-amyloid 1-42/1-40 have been shown to stabilise toxic oligomeric species and to correlate with levels of neurodegeneration in AD while increases in β-amyloid 1-40 have been suggested to prevent amyloid deposition (Qiu et al. 2015). The neurotoxic effect of β-amyloid could be conferred through the generation of reactive oxygen species or the triggering of apoptosis through the activation of caspases as both have been observed *in vitro* (Troy et al. 2000; Cheignon et al. 2018). β-amyloid oligomers have been shown to damage synapse structure and function *in vitro* and have also been shown to impair memory function in rats. Additionally, higher levels of β-amyloid oligomers, as opposed to plaques, have been correlated with clinical AD (Selkoe and Hardy 2016).

1.5.2 α-synuclein in neurodegeneration

α-synuclein is a small cytosolic protein widely expressed in the brain, in particular in the regions of the *substantia nigra,* the hippocampus and the neocortex. It is coded for by the gene *SNCA* (Stefanis 2012). It is part of the synuclein family of proteins that share a similar sequence that in humans include β-synuclein and γ-synuclein (George 2002). α-synuclein was first identified as part of the non-amyloid component (NAC) of amyloid plaques in Alzheimer's disease. A variety of disease have been shown to have accumulated, intracellular aggregates of the protein. These diseases include, AD, PD, dementia with Lewy bodies, diffuse Lewy body disease and multiple system atrophy. The inclusions found in the cells of diseased patients are termed Lewy bodies (Wakabayashi et al. 2007).

1.5.2.1 Structure

The structure of α-synuclein has been of considerable interest as it can inform on the protein's function (Figure 1.4). It is a 14 kDa protein made up of 140 amino acids (Lashuel et al. 2013). Its N-terminus is lysine-rich and possesses an amphipathic nature, while its acidic C-terminus is disordered. Its middle region, also termed the NAC region is highly hydrophobic.

Even though the association of α-synuclein with neurodegeneration has been extensively studied, neither its physiological function nor its functional conformation have been agreed upon. Traditionally α-synuclein has been viewed as a monomer. The monomeric form of α-synuclein is thought to exist in a disordered state which has high conformational flexibility (Breydo et al. 2012). α-synuclein is largely considered to be a cytosolic protein. However, its N-terminus has been shown to readily bind to lipid membranes and make the protein adopt a more helical conformation, enhancing its tendency to form multimers (Dikiy and Eliezer 2012). Membrane binding has been observed in up to a third of α-synuclein in the cell (Visanji et al. 2011). α-synuclein binds lipids with acidic headgroups and favours smaller in size vesicles (Davidson et al. 1998). It has been found to localise to presynaptic vesicles, mitochondrial membranes, and sites of ER-mitochondria contact (Snead and Eliezer 2014; Guardia-Laguarta et al. 2015).

A newer proposition about the native form of α-synuclein has been that it may form tetramers. 58kDa helically folded α-synuclein tetramers have been detected in human brain samples when analysed under non-denaturing conditions or cross-linked (Bartels et al. 2011). Tetramers have been reported to be abundant in freshly biopsied brain samples and are assumed to be non-toxic (Dettmer et al. 2015). Other studies have disputed these findings and reported a primarily monomeric form of α-synuclein in brain tissue and cell lines and explained that the reported tetramers were most likely due to the tendency of α-synuclein to form extended conformations (Fauvet et al. 2012; Lashuel et al. 2013; Burré et al. 2013). The native form of α-synuclein could be a monomer or a tetramer with evidence supporting both theories, but neither of which being widely accepted. Some data suggest that there exists an equilibrium between monomers and tetramers (Dettmer et al. 2016). It is possible that α-synuclein exists in multiple conformational and oligomeric forms that change depending on the cellular environment (Mor et al. 2016).

α-synuclein has been shown to bind metals such as copper, manganese and iron *in vitro* (Brown 2009). Different studies have identified different numbers of copper binding sites (Ranjan et al. 2017; Xiaoyan Wang et al. 2010; Davies et al. 2011). They have been found at the N-terminus, at H50, and with the C-terminus possibly coordinating copper as well but with lower affinity (Rasia et al. 2005; Brown 2009; McDowall and Brown 2016). The N-terminus copper binding site has been found to disappear upon α-synuclein N-terminal acetylation which is thought to occur *in vivo* with the protein still being able to bind copper at H50 (Mason et al. 2016).

Iron has also been shown to interact with the C-terminus of α-synuclein in two binding sites, with a particular affinity for the ferric form (Fe^{3+}) (McDowall and Brown 2016). The affinity of α-synuclein for iron and other metals can be altered through phosphorylation, where Fe^{2+} but not Fe^{3+} binds much more readily after phosphorylation at Y125 and S129, (Lu et al. 2011; Duce et al. 2017). Phosphorylation at S129 is thought to occur in DLB (Anderson et al. 2006). The interaction of α-synuclein with iron and copper has been suggested to play a role in α-synuclein oligomerisation (Lu et al. 2011; Carboni and Lingor 2015; McDowall and Brown 2016). Metal interactions are thought to play a major role in α-synuclein biology while at the same time α-synuclein is thought to play a major part in iron homeostasis (Lingor et al. 2017; McDowall and Brown 2016).

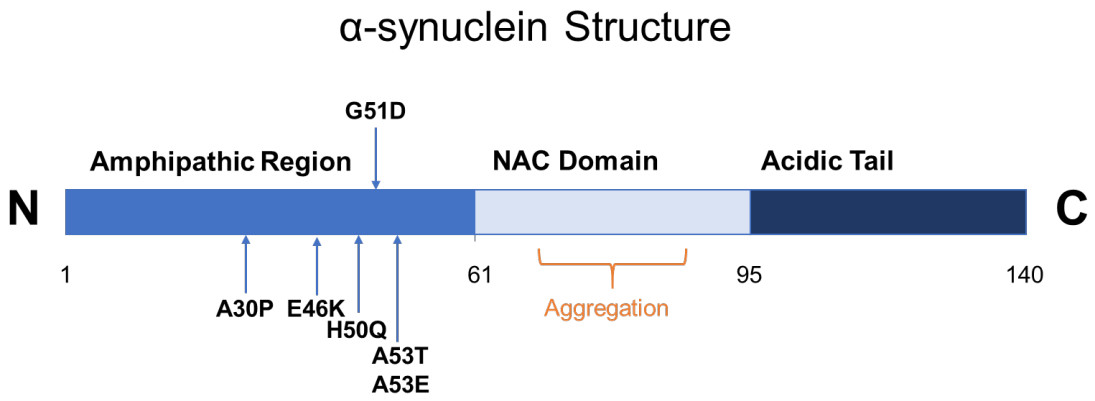

Figure 1.4 Structure of α-synuclein. Shown here is a schematic of the structure of α-synuclein including its' three main domains: The N-terminus amphipathic region, the middle NAC domain and the C-terminus acidic tail. The NAC domain is the one implicated in the aggregation of α-synuclein while all the common disease related mutations of α-synuclein occur in the N-terminus. Adapted from Xu and Chan 2015.

1.5.2.2 Function

Theories about α-synuclein function include it acting as a dopamine release mediator and it being a ferrireductase. As it is present in presynaptic terminals it was suggested that it is involved in synaptic function (Sidhu et al. 2004). α-synuclein may act as a chaperone in soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex assembly by directly binding synaptobrevin-2, facilitating the release of dopamine from the synapse (Burré et al. 2011). These observations were based on the monomeric form of α-

synuclein, but there is data suggesting that a multimeric form that is bound to lipid membranes is necessary to fulfil this function (Burré et al. 2014). On the other hand, α-synuclein oligomers have also been linked to preventing SNARE-mediated vesicle docking (Choi et al. 2013).

Another theory for α-synuclein function is that it is an enzyme (Davies et al. 2011). It has been found to reduce iron both *in vitro* and *in vivo* and that its active form is actually a tetramer (Bartels et al. 2011; McDowall et al. 2017). α-synuclein interacting with copper has been shown to be essential to ferrireductase activity, thus presenting a theory that incorporates a physiological function for copper binding. Increased ferrireductase activity has been detected in rat brains overexpressing α-synuclein. It is possible that ferrireductase activity in dopaminergic neurons can be neurotoxic as it was shown to increase cells' sensitivity to DOPAL (McDowall et al. 2017). Moreover increased ferrireductase activity of α-synuclein has been linked to increased production of β-amyloid in α-synuclein overexpressing cells, possibly as a result of increased oxidative stress (Roberts et al. 2017).

The apparent multifunctionality of α-synuclein could be linked to its conformational flexibility. It does seem like α-synuclein's role in the cell is more of a supportive rather than essential molecule, as knockout organisms are viable possibly due to some functional redundancy with the other synucleins. α-synuclein null mouse models are also resistant to the neurotoxic effects of MPTP and LPS. Conversely, both excessive and overly reduced levels of α-synuclein result in impaired neuronal function (Stefanis 2012). α-synuclein's properties may be more related to long-term neuronal viability and become more visible under conditions of stress. Perturbations in α-synuclein localization, production and clearance can prevent it from fulfilling its neuroprotective function and instead form toxic oligomeric or fibrillar species.

1.5.2.3 Aggregation

α-synuclein is clearly a central molecule to the pathology of PD and other synucleinopathies. It most famously aggregates to form the main component of Lewy bodies and the non-amyloid component in AD amyloid plaques in the brain. The brain is not the only location α-synuclein has been found to aggregate with recent studies proposing that α-synuclein aggregation is initiated in the gut (Liddle 2018; Klingelhoefer and Reichmann 2015). α-synuclein has been shown to be expressed by enteroendocrine cells that connect to enteric nerves which allow α-synuclein aggregation to travel up the vagus nerve to the brain (Chandra et al. 2017). The types of aggregate found in Lewy bodies are mostly fibrils (Spillantini et al. 1997). Apart from these best-known types of aggregates α-synuclein can form protofibrils and a multitude of oligomers including small dimers, trimers and tetramers and bigger spheres, chains or rings. α-synuclein is thought to be constantly cycling between monomeric and oligomeric forms and for oligomers to assemble into fibrils through a nucleated polymerization mechanism. α-synuclein aggregation can be enhanced by the interaction with lipids such as those in membranes in the cell, but it has been shown to occur in both the cytosol and in association with lipid membranes (Lashuel et al. 2013; Galvagnion et al. 2015). The N-terminus region is the

part of α-synuclein that interacts with lipid membranes, while the NAC region is central to aggregation (Bisaglia et al. 2006). In fact, *in vitro* studies have shown that deleting that region greatly diminished α-synuclein's ability to oligomerize and form fibrils (Lashuel et al. 2013). β and γ-synuclein differ from α-synuclein in this region and have not been observed to aggregate in disease. In contrast, the C-terminal section of α-synuclein is thought to have a stabilising function and deleting it results in molecules with a greater inclination to aggregate (Serpell et al. 2000). C-truncated species of α-synuclein that can be produced by proteases also show increased aggregation (Sevlever et al. 2008). In a mouse model of α-synuclein aggregation passive immunization against the C-truncated species prevented aggregation and ameliorated motor symptoms (Games et al. 2014). Another argument for the central role of α-synuclein aggregates in disease is that mutations in SNCA have been identified to cause rare familial forms of PD. The most common polymorphisms A30P, E46K, A53T and G51D are connected to early onset of the disease and H50Q is linked to a later presentation of symptoms. All of these mutations have a destabilising effect on the N-terminus and are thought to increase the formation of α-synuclein aggregates (Flagmeier et al. 2016; Brucale et al. 2009).

Metal interaction has been shown to induce α-synuclein aggregation, where both the presence of copper and iron can result in the increased formation of α-synuclein aggregates (Uversky et al. 2001). In fact, in copper-free environments α-synuclein is unable to aggregate (Xiaoyan Wang et al. 2010).

1.5.2.4 Toxicity

The mechanism of toxicity of α-synuclein has been the subject of a lot of research and some progress has been made. Lewy bodies, the pathological feature of PD and DLB were originally thought to confer the toxic effect but in fact high levels of Lewy bodies have been found in a large proportion of aged non-demented individuals, thus suggesting that Lewy body accumulation may be a factor of age (Schulz-Schaeffer 2015). Additionally, Lewy bodies in PD have been found to appear after the onset of pathology and neuronal death. Moreover, the presence of Lewy bodies inside neurons didn't correlate with neuronal cell death in the *substantia nigra* (Tompkins and Hill 1997).

The potential native tetrameric form of α-synuclein is resistant to aggregation (Bartels et al. 2011). It is possible that processes related to ageing or environmental exposure, as well as mutations destabilise the α-synuclein tetramers and can lead to the formation of toxic oligomeric species or aggregates. The PD causing mutations A53T and E46K have also been shown to destabilise tetramers by increasing the energy barrier to tetramerization (Dettmer et al. 2015; L. Xu et al. 2018). α-synuclein has been shown to be able to oligomerise in laboratory conditions by the modulation of a myriad of factors including stirring, shaking and also adding ethanol, iron, copper or dopamine. The nature of the species of α-synuclein oligomers that are neurotoxic is evasive, but it is generally agreed that they exhibit an increased prevalence of a β-sheet secondary structure (Wong and Krainc 2017). A cell culture model of α-synuclein aggregation showed that copper is required for the neurotoxicity of α-synuclein and the type of oligomer that conferred the toxicity was stellate in shape with a β-sheet structure (Wright et al. 2009).

Elevated levels of soluble α-synuclein oligomers have been found in the brains of sufferers of DLB (Paleologou et al. 2008). In animal models, genetic variants of α-synuclein that are prone to forming oligomers are more neurotoxic than ones prone to aggregating (Winner et al. 2011). Large α-synuclein oligomers have been shown *in vitro* to decrease the assembly of the SNARE complex, a proposed function of α-synuclein and thus acting to inhibit dopamine release at the synapse (Choi et al. 2013). α-synuclein oligomers have been shown to cause increased glial activation and neuroinflammation through TLR4 receptor activation (Fellner et al. 2013). The pore-like structure that α-synuclein oligomers can assume can result in their incorporation into the membrane causing disrupted Ca^{2+} in neurons. Additionally, soluble oligomers can cause mitochondrial dysfunction, increase in ROS, impaired cytoskeletal formation, endoplasmic reticulum stress and impaired protein degradation (Roberts and Brown 2015). This way they can not only directly confer neurotoxic effects but also perpetuate decreased α-synuclein clearance.

How α-synuclein comes to accumulate in sporadic synucleinopathies is a question of significant importance. α-synuclein gene duplication and triplication has been shown to be the cause of rare familial PD thus pointing to the fact that increased levels of the protein are a disease-driving mechanism (Devine et al. 2011). Studies have found no increase in α-synuclein mRNA in sporadic PD or in DLB compared to controls, therefore it's possible that the accumulation of α-synuclein in those cases is due to impaired clearance mechanisms (Quinn et al. 2012; Su et al. 2017). Major protein clearance mechanisms such as the ubiquitin-proteasome system and autophagy that have been shown to degrade α-synuclein are dysregulated in sporadic and hereditary synucleinopathies (Cuervo et al. 2004; Ghavami et al. 2014). In DLB α-synuclein accumulation has been linked to reduced expression of α-synuclein degrading proteases neurosin (kallikrein-6) (extracellular) and calpain-1 (intracellular) (Miners et al. 2014; Iwata et al. 2003). Cathepsin-D is another protease known to process α-synuclein that acts in the lysosome, with partial proteolysis resulting in the formation of C-terminal truncated species more prone to aggregation, but its deficiency is has also been shown to result in increased aggregation (Crabtree et al. 2014; Cullen et al. 2009; Sevlever et al. 2008). Glucocerebrosidase is another lysosomal enzyme implicated in α-synuclein aggregation, where loss of function results in an accumulation of its substrate which in turn facilitates α-synuclein aggregation (Mazzulli et al. 2011). MMP-3 processes α-synuclein in the extracellular space, is secreted by microglia in stress conditions and also results in C-terminal truncated species (Choi et al. 2011; Sung et al. 2005). Modulating the levels of these enzymes is considered a potential avenue for preventing neurotoxicity in PD and DLB (Wong and Krainc 2017; Park and Kim 2013).

What all the current knowledge points to is that α-synuclein is the toxic molecule behind synucleinopathies and that understanding all the factors that lead to the formation of toxic oligomers in the brain will be crucial to both the prevention and the treatment of these diseases.

For both α-synuclein and β-amyloid, ineffective clearance mechanisms seem to be central to accumulation and further disease development. Investigating the underlying reason for this ineffective clearance could be key to preventing sporadic age-related neurodegenerative disease. As the world population ages, it is becoming increasingly

imperative to not only manage the symptoms of these neurodegenerative conditions as and when they appear, but also to find ways to prevent them and reverse them. In order to achieve that it is important to recognise that aging plays a central role in the development of neurodegeneration.

1.6 Aging

Aging is a process that occurs in living organisms. It is characterized by changes on the physiological, cellular and molecular level that result in the impairment of many functions of the organism. Death rates in many organisms including humans increase as a function of age. Additionally, humans are one of the few animals whose lifespan extends far beyond their fertile years. In humans, aging is a risk factor for many diseases that include: heart disease, pulmonary disease, cancer, and neurodegenerative diseases.

Unlike humans some organisms such as some turtle species or naked mole rats do not exhibit a decline in fitness with age, a phenomenon called negligible senescence (Kogan et al. 2015). Others examples such as the hydra display indefinite stem cell division possibly due to the transcription factor FOXO activity (Boehm et al. 2012). Research also suggests that lobsters' continuous expression of the enzyme telomerase protects them from accumulating DNA damage and prevents them from suffering any age-related changes in fitness (Klapper et al. 1998).

1.6.1 Theories on the cause of aging

The underlying reasons to the aging process have not been fully elucidated and many competing theories have been proposed over the years.

Firstly, it is possible that life-spans are evolutionarily pre-programmed. Some have argued that ageing is evolutionarily beneficial while others believe it is a side-effect of sexual selection. The mutation accumulation theory proposes that random germline mutations accumulated over time that only affect an organism later in life do not get selected out of the gene pool as most animals in nature don't survive long enough to reach old age (Kirkwood and Austad 2000). However, this theory does not account for differential gene expression in different tissues and different time points. The disposable soma theory states that organisms focus more energy and resources on growth and reproduction in early life due to evolutionary pressure and neglect cellular repair and maintenance which results in aging (Kirkwood 1977). This theory however doesn't explain the extended lifespans of many organisms as a result of caloric restriction. The antagonistic pleiotropy hypothesis suggests that a gene can have an effect on multiple traits some of which are beneficial and some detrimental (Williams 1957). It is possible that genes that favoured an individual's fitness in early life were selected during the evolutionary process. This comes as a trade-off with later age impairment after the individual has procreated. Aging would be caused when the benefits of those genes are shown early in life and the costs later. However, from the genes that have been identified to control aging and are conserved in different species not many have been identified to bring early-life benefits to fitness or reproductive ability. The effectiveness of the molecular mechanisms that regulate tissue maintenance and repair correlates with the overall lifespan of a species and more precisely with the time it takes for them to reach maturity. These mechanisms are complex and involve a multitude of interacting molecules. It is possible that they are controlled by a master switch capable of sensing outside conditions and thus modulating lifespan. The eventual failure of these systems to maintain cellular viability over a longer lifespan could therefore be passed on to the germline as it would not prevent the individual from

reaching adulthood and procreating (Kirkwood and Austad 2000). An argument against the pre-programmed lifespan theory is that in twin studies the genetic factor in heritability of human lifespans has been found to be only about 20-30% and to increase with age (Wyss-Coray 2016). This means that the majority of factors that determine the length of the human life are external such as their lifestyle, surroundings and exposure to toxins.

External factors can impact an organism's lifespan by inducing changes in their genome. Epigenetic changes in DNA occur over an organism's lifespan which can lead to differential expression in many genes related to viability, cell division and dealing with cellular damage (López-Otín et al. 2013). Dysregulation of these processes is considered a hallmark of aging and could explain the differences in the transcriptome between young and aged cells (Horvath and Raj 2018). However, linking DNA methylation changes to actual changes in expression has proved challenging due to the dynamic nature of RNA expression (Sen et al. 2016). Additionally, it has not been determined how these changes come about and whether they are the cause of or an effect of aging.

Natural accumulation of DNA and other macromolecular damage that results in the failure of many biological systems is another theory commonly used to explain aging (Gensler and Bernstein 1981). Indeed, a correlation between DNA damage and age has been shown a meta-analysis of human aging studies (Soares et al. 2014). The types of DNA damage that can accumulate include: point mutations, translocations, gene disruption and chromosomal abnormalities. They can be caused by oxidative stress, telomere shortening or just random chance errors that escape DNA proofreading and repair mechanisms (Maynard et al. 2015). Telomeres are structures in the ends of chromosomes that stabilize them and protect the integrity of DNA during cell division. Telomere shortening occurs every cell division and leads to chromosome instability and cellular senescence (Jiang et al. 2007). However, a mouse model of loss of telomerase activity doesn't replicate all symptoms of aging (Rudolph et al. 1999). While on one hand telomerase deficiency has been linked to the premature development of diseases, telomere length in human blood cells has been found to be heterogenous with a possible telomere maintenance mechanism being at play in individuals with shorter telomeres (Nordfjäll et al. 2009; Svenson et al. 2011). It has been suggested that this pre-programmed limit on cellular division has evolved to suppress cancer (Cerella et al. 2016).

DNA replication and translation into proteins is a process that occurs countless times over an organism's lifetime and seems to be affected by ageing, with an accumulation of aberrant DNA polymerases and an increase in the mis-incorporation of nucleotides and amino acids (Milholland et al. 2017). It is thought that this results in an increase in protein errors and thus fundamental disruption in metabolic processes, leading to more damage and death (Rattan 2006). Organismal longevity clearly requires translational fidelity and accuracy of translation has been found to correlate with lifespan in rodents (Ke et al. 2017). The evidence of an age-related increase in protein errors has been largely unconvincing but error accumulation at a steady pre-determined rate could result in the same detrimental effects. Additionally it has been proposed that age-related accumulation in somatic mutations in gene regulatory regions of DNA rather than protein encoding ones can have the same results on the organism but that accumulation is yet to be proven experimentally (Milholland et al. 2017).

The free radical theory of aging postulates that cells accumulate free radical damage over an organism's life span (Harman 1956). Reactive oxygen species (ROS) which include free radicals are thought to act in biological systems to cause damage. ROS include oxygen radicals such as superoxide, and hydroxyl and nonradical oxidizing agents such as hydrogen peroxide among others (Bayr 2005). One of the main effects if increased levels of ROS during aging is thought to be oxidative damage (Liochev 2013). Oxidative damage can be induced by various environmental sources such as UV light, ionising radiation and toxins such as metals; and can cause not only DNA damage but damage to other cellular structures as well (Birben et al. 2012). ROS are also produced by mitochondria, the endoplasmic reticulum, peroxisomes and the cell membrane and play roles in normal cell metabolism (Phaniendra et al. 2015). Levels of ROS are constantly managed by enzymes such as superoxide dismutase and catalase but dysfunction in these processes can result in impairment of their protective functions (Lund et al. 2009; Selvaratnam and Robaire 2016). ROS can react with DNA and RNA where mitochondrial DNA is particularly vulnerable (Brunk and Terman 2002). ROS can also cause lipid peroxidation in membranes and react with amino acids in proteins. Moreover, ROS have been implicated in endoplasmic reticulum stress. Accumulation of ROS damage is thought to lead to apoptosis. On the other hand the role of ROS as important cell signaling molecules has been emerging and casts doubt on the free radical theory of aging (Ray et al. 2012; Hancock et al. 2001). Additionally, studies have shown that caloric restriction, which results in elevated oxidative stress can have a positive effect on lifespans (Fontana et al. 2010). It is possible that it is not the overall increase in intracellular ROS that initiates the negative effects of aging but the cells' declining ability to maintain them at an appropriate level (Finkel and Holbrook 2000).

1.6.2 The molecular signature of aging

Understandably, great interest has been shown in the molecular causes of aging, with the idea that identifying which cellular processes are disrupted in an aged organism can lead to the amelioration or reversal of aging in humans. Aging proceeds in a similar manner in many organisms. The hallmarks of aging include genomic instability, telomere shortening, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (López-Otín et al. 2013).

An avenue for investigation of the molecular players in ageing is the study of longevity and what genetic variants confer a longer life in humans (Marques et al. 2010). Genome-wide association studies have had limited success in that field by reproducibly linking only APOE variants and FOXO3a variants with longevity (Broer et al. 2015; Nebel et al. 2011; Walter et al. 2011; Broer and van Duijn 2015). The difficulty in identifying single "longevity genes" through GWAS is due to the complexity of traits such as longevity and aging. Pinpointing which common variants with small individual effects interact together to produce a larger effect on longevity is a possible step forward. Another possible avenue in the study of longevity is identifying ways to measure changes in the larger pathways implicated in aging.

The hallmarks of aging manifest through the altered functioning of many molecular pathways in the cell. The idea that an aged organism can be identified by reproducible changes in gene expression has been investigated in a multitude of gene-expression studies (Kent et al. 2012; Van den Akker et al. 2014; Glass et al. 2013; Park and Prolla 2005; Welle et al. 2003; Lee et al. 2000; Peters et al. 2015; Bryois et al. 2017; Ardlie et al. 2015).

A major analysis of changes in gene expression with age has uncovered large clusters of genes affected by age in a variety of tissues (Yang et al. 2015). The study highlighted that most tissues exhibited age-related mitochondrial dysfunction. They also commonly observed changes in DNA repair pathways, apoptosis, inflammation and the electron transport chain. Those findings corroborated many other studies that implicate these processes in aging in both humans and animal models. The aging signature of some tissues especially the nervous tissue and arteries correlated best with chronological age. In fact, pathways linked to age-related ND such as AD, PD and Huntington's were significantly altered in multiple tissues. An additional finding was that genes implicated in tissue-specific diseases were over-represented in the same tissues, with pulmonary function markers downregulated in the lung, obesity associated genes elevated in adipose tissue and immune and inflammatory genes elevated in blood and arteries.

Even though a lot of literature has been published on the topic it has proven difficult to identify universal genetic biomarkers for biological age. That is probably due to the great complexity through which the genotype and the environment interact to produce a phenotype. Measuring the levels of proteins and metabolites in the body is an alternative avenue through which biomarkers can be identified. Protein and metabolite levels have been found to change with age in both model and non-model organisms and in humans (Menni et al. 2015; Hoffman et al. 2017). Metabolic studies of aging have also moved more towards identifying pathways that are disrupted rather than individual molecules.

One way to look at aging is through the study of disrupted cellular networks. In many biological pathways there are a small number of genes that have many interacting partners. Disruption in the activity of any of these genes results in disruption in the whole network. Some genetic pathways identified in high-throughput gene expression studies have been previously linked to aging through studies on life extension and longevity. A well-known life-extending factor in model organisms is caloric restriction which alters nutrient sensing pathways in the cell (Haigis and Yankner 2010). A small number of highly conserved pathways, namely the FOXO3a signalling pathway (mediated by insulin/IGF or AMPK), the target of rapamycin (TOR) pathway and sirtuin signalling are implicated in nutrient sensing, stress response, chromatin remodelling and DNA damage response. The proteins that control these pathways have a very large number of interacting partners and targets and can be manipulated to modulate survival and longevity. Therefore, they are thought to be key regulators of aging.

Protein	Functions	Aging changes
FOXO3a	Tumour suppressor, oxidative stress sensor, can both enhance survival and be pro-apoptotic	More pro-apoptotic signalling
mTOR	Inhibits autophagy, increases protein, nucleotide and lipid synthesis	Increased signalling
SIRT1	Histone deacetylation, DNA repair, anti-inflammatory, pro-survival signalling	Reduced signalling
AMPK	Sensor for nutrient scarcity, activates autophagy, connected to SIRT1 with a positive feedback loop	Reduced signalling
PGC-1α	Activates mitochondriogenesis, improves antioxidant defences, stimulates fatty acid oxidation. Activated by SIRT1, acts with mTOR	Reduced signalling
ATM	Major DNA repair regulator, works in conjunction with SIRT1, inhibited by mTOR	Reduced signalling
IGF-1	Nutrient sensor enables growth and survival, triggers FOXO inhibition and mTOR activation through AKT	Reduced signalling

Table 1.1 Proteins associated with aging. The proteins listed in this table alter their activity in the aging organism and interact with each other to cause the detrimental changes seen in the aging cell. The first column lists the protein or protein family implicated, the second one summarises their functions in the cell and how they have been found to interact with the others and the third summarises how their signalling changes with age.

1.6.2.1 SIRT1

Sirtuins are a family of proteins that possess a NAD-dependent deacetylase or a ADP-rybosyltransferase function. They are linked to multiple hallmarks of aging, namely genomic instability, deregulated nutrient sensing and epigenetic alterations (López-Otín et al. 2013). Studies in budding yeast, *C. elegans* and *Drosophila* first demonstrated the potential importance of sirtuins to aging (Rogina and Helfand 2004; Tissenbaum and Guarente 2001; Kaeberlein et al. 1999). Overexpression of sirtuins in those organisms was found to increase lifespan. After some conflicting findings a more nuanced role for sirtuins in lifespan regulation has been emerging (Whitaker et al. 2013; Burnett et al. 2011; López-Otín et al. 2013). Mammals possess 7 sirtuin proteins SIRT1-7 where SIRT1 has been the best studied. SIRT1 expression has been found to decline with age in many tissues. Additionally, in mouse models of accelerated ageing, SIRT1 levels are found to decline with age as well (Gong et al. 2014).

A major function of SIRT1 involves DNA repair as it can deacetylate DNA repair proteins and NBS1, a DNA double strand break sensor (Yuan et al. 2007; Fan and Luo 2010; Jeong et al. 2007). SIRT1 activity in DNA repair is thought to be ataxia telangiectasia mutated serine/threonine kinase (ATM) -dependent and ATM is found to be globally

reduced in aging (Dobbin et al. 2013). SIRT1 has also been implicated in epigenetic modification of chromatin through histone deacetylation (Zhang and Kraus 2010).

SIRT1 is also plays a role in maintaining proteostasis. Targets of SIRT1 include autophagy related proteins, which are activated in starvation conditions. Autophagy is increased in times of nutrient deprivation as a way to create a source of energy and of free amino-acids to be used in protein synthesis. Maintaining autophagic flux is central process for cellular stress response and longevity. Thus, reduced levels of SIRT1 signalling in aged organisms can result in inadequate protein turnover.

SIRT1 activity can be reduced by a high-fat diet but stimulated by caloric restriction. SIRT1 deacetylates many proteins related to metabolism and stress response including nuclear factor kappa B (NFκB), p53, FOXO transcription factors and many others. An important target of SIRT1 is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), an important stimulator of mitochondrial biogenesis and regulator of metabolism. In animal models SIRT1 deficiency results in impaired energy metabolism, damaged mitochondria and elevated ROS. Elevated SIRT1 expression results in improved metabolic parameters, but potential lifespan extension caused by SIRT1 may only be conferred in stress conditions (Bordone et al. 2007).

Other sirtuins have also been implicated in lifespan extension. Mice deficient in SIRT6 have a reduced lifespan and ones with elevated SIRT6 levels live longer than controls. Those effects are thought to be conferred by a reduction in IGF-1 signalling. SIRT3 has also been found to have beneficial effects on the regenerative capacity of cells *in vitro*.

1.6.2.2 mTOR

Mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that is implicated in cellular senescence and age-related disease. The mTOR pathway inhibits autophagy and therefore has an essential role in protein homeostasis. Its activity can be attenuated by rapamycin but also in conditions of caloric restriction. Mammalian mTOR signalling has been found to be reduced in a mouse model of longevity. Additionally, inhibiting mTOR signalling can extend the lifespan of a variety of model organisms (Johnson et al. 2013). The targets of the mTOR pathway include not only autophagy but also cell growth, protein synthesis, ribosomal biogenesis and metabolism. In mammals the mTOR pathway is represented by two distinct protein complexes, mTORC1 (comprised of mTOR, RAPTOR, PRAS40 and MLST8) and mTORC2 (comprised of mTOR, RICTOR, MSIN1, PROTOR and MLST8). Downregulating the mTORC1 pathway in mice results in an extended lifespan. Key downstream targets of the mTORC1 include S6 kinase and eIF4E-binding protein 1 (Gingras et al. 1998; Magnuson et al. 2012). mTORC1 has also been found to interact with PGC-1α (Cunningham et al. 2007). Another interesting downstream effector of the mTOR pathway is the inhibition of ATM (Shen and Houghton 2013). Loss of ATM signalling in post-mitotic neurons in the genetic disorder ataxia telangiectasia results in neurodegeneration. Additionally, the mTOR pathway has also been found to control cell proliferation by

regulating the FoxO3a transcription factor via SGK1 kinase. Inhibiting mTORC1 signalling resulted in pro-survival and proliferation signalling by FOXO3a (Mori et al. 2014).

Interestingly, alleles implicated in age-acceleration in the cerebellum localize near one of the mTOR complex subunits MLST8 (Lu et al. 2016). Autophagy is implicated in both aging and neurodegeneration where it is found to be beneficial for cellular survival and longevity (Menzies et al. 2015). Deficiency in autophagy related genes in mouse models results in protein aggregation and neurodegeneration. Blocking mTOR signalling increases lifespan possibly through increasing autophagy.

The current level of knowledge suggests that aging is unlikely to be caused by a single mechanism. It seems to be a multifactorial process that is affected by changes in many different systems. Additionally, aging in mammals is thought to proceed in an organ-specific manner (Yang et al. 2015). However, loss of protein homeostasis is a major hallmark of the aging organism. Proteostasis is constantly maintained at the cytosolic, organelle, cellular, intercellular and organ level. Defects in protein chaperones, the ubiquitin proteasome system and autophagy all contribute to the accumulation of misfolded and possibly toxic proteins with age. In a multitude of model organisms, interventions that stimulate these systems result in increased lifespans. The fact that the Sirtuin, FOXO3a and mTOR pathways have all been found to contribute to maintaining proteostasis and the fact that some of the most major age-related diseases have been linked to loss of proteostasis all point to a potential central role changes in these proteins play in age and disease (Morimoto and Cuervo 2014; Kaushik and Cuervo 2015).

1.6.3 Cellular senescence and the senescence associated secretory phenotype

Cellular senescence is a process that results in an arrest in cellular proliferation. It is thought to contribute to the process of aging and age-related disease. It was first identified *in vitro* where primary cells would divide a limited number of times known as the Hayflick limit (Hayflick and Moorhead 1961). The arrest in cell cycle is triggered by the shortening of telomeres and increased proteotoxicity which triggers the activation of the DNA damage response protein ATM which in turn activates tumour suppressor proteins such as p16 (Childs et al. 2015). *In vitro* senescent cells are characterised by an increased size, resistance to apoptosis, increased senescence-associated β-galactosidase activity (SA-β-GAL) and a senescence-associated secretory phenotype (SASP) (Dimri et al. 1995; Hampel et al. 2005; Ryu et al. 2007; Coppé et al. 2010). The SASP is characterised by increased transcription of inflammatory cytokines (IL-1α,IL-1β, IL-6, IL-8 among others) proteases (MMPs and serine proteases) and increased release of NO and ROS while on the other hand, anti-inflammatory cytokines and proliferation and differentiation factors remain unchanged or are reduced (Ghosh and Capell 2016; Coppé et al. 2010). SASP is largely caused by NFκB and p38 activation triggered by the response to genomic instability and mediated by mTOR increase and SIRT1 reduction in activity (Salminen et al. 2012; Ghosh and Capell 2016). The alteration in the secretory phenotype of senescent cells has been shown to alter the intercellular environment and to cause senescence in neighbouring cells *in vitro* (Nelson et al. 2012).

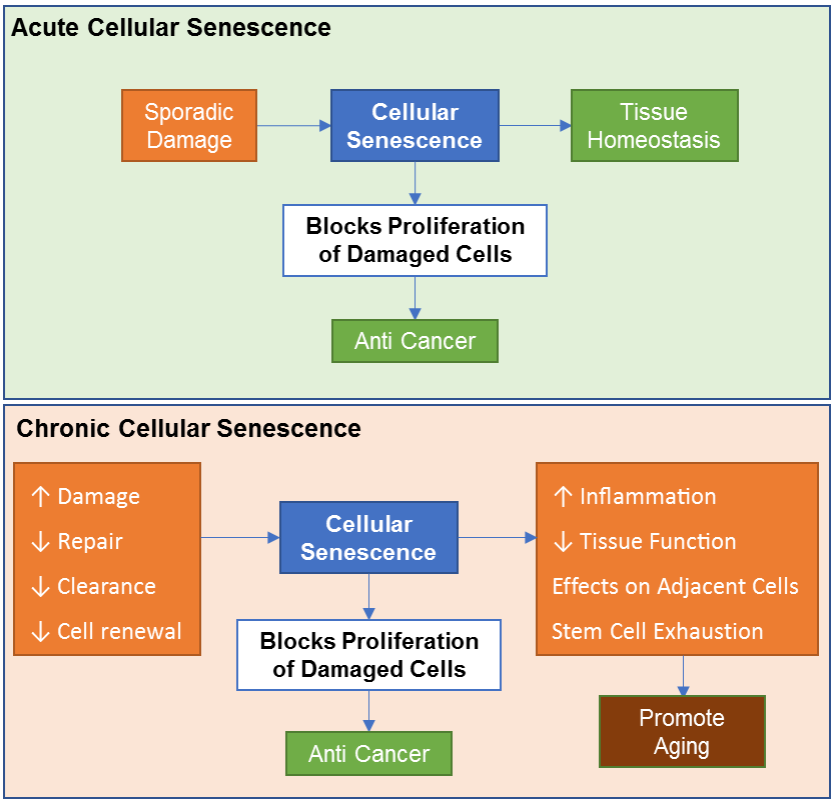

Figure 1.5 Comparison of acute and chronic cellular senescence. Acute cellular senescence occurs in young organisms and maintain tissue homeostasis and are antitumorigenic by preventing cells that suffered genomic damage from proliferating. Chronic senescence occurs in older organisms and is the result of an accumulation of damage and can result in increased numbers of senescent cells due to inefficient clearance mechanisms. Those senescent cells can have multiple negative effects on tissue homeostasis. Adapted from López-Otín et al. 2013.

In vitro cellular senescence can be induced though irradiation, RAS overexpression or chemotherapeutics (Childs et al. 2015). *In vivo* cellular senescence is commonly observed and can be separated into three categories: acute, embryonic and chronic (Figure 1.5). Acute and embryonic senescence have an important role in development and wound healing and seem to be programmed and beneficial to the organism. Chronic senescent cells are the type that presents in human aging and is linked to the development of age-related disease as they can persist in tissues for a long time and potentially propagate through paracrine signalling (Nelson et al. 2012; van Deursen 2014). Chronic senescence can be caused by slow accumulation of damage to macromolecules such as protein aggregation, misfolding and DNA damage over time (Childs et al. 2015). The SASP of chronic senescent cells is thought to be more heterogeneous as it has been suggested that acquiring the senescent phenotype in aging is a gradual process (van Deursen 2014). Senescent cells *in vivo* can be identified by markers such as SA-β-GAL and p16 and have been shown to accumulate in aged tissues (Geng et al. 2010; Gruber et al. 2007; Krishnamurthy et al. 2004). Senescent cells have an important role in cancer with their secretory phenotype being both pro- and anti-tumorigenic but allowing them to be targeted for immune clearance (Kang et al. 2011; Cerella et al. 2016). Thus, the targeting of senescent cells for therapeutic elimination in ageing can be difficult because the same processes that trigger their senescence also work to protect from tumour development. It

is thought that cellular senescence is a protective mechanism that prevents cancers but in later age can be detrimental (Cerella et al. 2016). The processes that link cellular senescence and aging are SASP factors resulting in a diminished ability to repair damage to tissues and to losing stem cells that are needed for replenishing and renewing tissues to senescence (Childs et al. 2015)

1.6.4 Aging in the nervous system

In the human brain, aging shows some characteristics that are similar to the rest of the organism such as DNA damage, altered DNA methylation, increased inflammation, loss of protein homeostasis, metal dyshomeostasis and deregulated nutrient sensing (López-Otín et al. 2013; Wyss-Coray 2016).

Accumulation of DNA damage is a major issue in the nervous system as neurons are post-mitotic cells (Chow and Herrup 2015). That means they are unable to carry out some routes of DNA repair that dividing cells are such as homologous recombination. The amount and patterns of DNA damage and repair in neurons are heterogeneous and are thought to start in development and are often the result of random external factors but also high transcription rates due to neuronal activity (Suberbielle et al. 2013). DNA methylation is another factor that is affected by aging in the brain, with the changes being consistent with the Horvath epigenetic clock, a generalised model that can predict the age of a tissue based on its methylation status with the exception of the cerebellum which is epigenetically “younger” than other brain tissues as it contained fewer age-related methylation markers than other brain areas (Horvath et al. 2015). In the aging brain genes that increase inflammation are preferentially activated in astrocytes and microglia and different communication molecules such as pro-inflammatory cytokines are secreted (Spittau 2017; Salminen et al. 2011). That could be due to epigenetic changes that change the secretory phenotype of the cells or due to the cells suffering protein homeostatic dysregulation for other reasons that switch them to a pro-inflammatory state. This pro-inflammatory state seems to contribute to the development of neurodegenerative disease.

Proteins implicated in ageing such as SIRT1 and mTOR have also been linked to the ageing of the brain. Declining SIRT1 signalling in the brain results in impaired energy metabolism and circadian rhythms but also reductions in neuronal plasticity. They have also been implicated in increased neuroinflammation caused by activated microglia (Braidy et al. 2015; Satoh et al. 2017). Impaired mTOR signalling in the brain leads to disrupted energy metabolism, mitochondrial function and autophagy all of which are affected in the aged brain (Perluigi et al. 2015). Inhibiting mTOR signalling has the potential to rescue neurons from age-related degeneration. Increased autophagy induced by inhibition of mTOR signalling can ameliorate cognitive deficits in aged mice (Yang et al. 2014).

Loss of protein homeostasis is a major hallmark in aging brains. That includes accumulation of protein aggregates such as amyloid plaques, Lewy bodies, neurofibrillary tangles and lipofuscin in cognitively healthy aged brains. These are connected to lysosomal dysfunction and autophagy disruption. The accumulation of lysosomal proteins in the brain and the presentation of abnormal mitochondria, autophagosomes, endosomes

and lysosomes in both neurons and glia is another piece of evidence of the inefficient protein degradation pathways seen in aging (Menzies et al. 2015; Nixon et al. 2005; Nixon et al. 2000; Brunk and Terman 2002). Major changes in the levels of key metabolites have been found in the brains of aged mice. Those include declining levels of NAD and increases in AMP/ATP and abnormal nucleotide synthesis (Ivanisevic et al. 2016). This is another example of how cellular homeostasis is disrupted in the aging brain.

Another hallmark of the aging brain is the accumulation of metals. Iron in particular has been reliably shown to accumulate in multiple brain regions. On top of that the dyshomeostasis of iron, copper and zinc have been implicated in ND. Even though metals are essential to cellular metabolism, in excess levels they can cause oxidative stress, ER stress, mitochondrial dysfunction, autophagy dysregulation and can activate apoptosis (Chen et al. 2016).

Considering all the ways in which cellular health is compromised in the aging brain it is not surprising that brain aging has also been linked to neuronal death and general loss of brain volume, a phenomenon that could be due to loss of myelin, glia or fluid as well (Peters 2006).

Research into the genomic signature of the aging brain is an important tool in understanding the age-related changes observed on the cellular level and to uncover what separates healthy brain aging and neurodegeneration. Many efforts have been made to document the ageing signature of the brain with findings generally pointing to the fact that it is heterogenous, with few gene expression studies having the same conclusions, possibly due to the heterogeneity of brain tissue itself. Genes involved in the aging of the brain are not necessarily affected in the aging of other systems. In one study only around $1/5^{th}$ of genes associated with age in the cortex or the cerebellum changed in the same direction as in whole blood (Peters et al. 2015) Gene expression studies in the human aged brain have found that pathways related to oxidative stress, immune activation and inflammation are upregulated in ageing while genes related to synaptic vesicle trafficking and calcium regulation were downregulated (Kumar et al. 2013). Network analysis has also shown that mitochondrial pathways are downregulated in aging in the human brain. Different regions of the brain have also been found to age differently. For example, lysosome metabolism and glucosaminoglycan degradation pathways have been implicated in the ageing of the cerebellum and the actin cytoskeleton, focal adhesion and tight junction pathways in the frontal cortex (Lu et al. 2004). The human pre-frontal cortex has also shown reductions in genes that play roles in mitochondrial function, vehicular transport and synaptic plasticity. On the other hand, genes related to inflammation, metal ion homeostasis and stress response were increased.

Broadly, the changes reported in the aging brain replicate the changes seen in neurodegenerative disease but to a smaller scale. Therefore, understanding the role that aging plays in the pathology of neurodegeneration may be central to understanding it.

1.6.5 Age as a risk factor for neurodegeneration

It is a widely known fact that the vast majority of neurodegenerative diseases, including AD, PD and ALS, affect predominantly the aged population. The mean age on onset for

PD is 70, for AD – 85 and for ALS – 65 (Hardiman et al. 2017; Hindle 2010). Therefore, some factors that change with aging must increase the susceptibility of the nervous system to disease. As an organism ages, the accumulation of defects in the variety of mechanisms mentioned above that are considered normal aging can reach a tipping point and lead to neurodegeneration. It is possible that individuals who suffer from neurodegeneration are more susceptible to aging than others. Interestingly, epigenetic changes to DNA have been found to show more signs of aging in neurodegenerative conditions. In a comparison of DNA methylation patterns between aged cortices with and without AD it was found that the AD brains were affected by epigenetic signatures of accelerated aging that correlated with loss in cognitive ability and histological markers of AD (Levine et al. 2015). Markers of increased immune system aging have been also detected in blood samples of PD patients (Horvath and Ritz 2015). Selective neuronal vulnerability is a phenomenon seen in neurodegenerative diseases but also in normal ageing. The genetic changes seen in aged vulnerable neurons have also been found in AD vulnerable neurons (Xinkun Wang et al. 2010). Additionally, changes in autophagy seen in aging have been heavily implicated in neurodegenerative disease. Mutations in Presenillin-1, linked to hereditary AD have been shown to impair autophagic flux and result in ineffective clearance of proteins. PINK1 and parkin, proteins that are mutated in hereditary PD are thought to promote mitochondrial autophagy, impairment in which results in dysfunctional mitochondria, generating high levels of ROS thus increasing oxidative stress (Haigis and Yankner 2010).

It is unclear which of the hallmarks of the aging brain actually control the process of aging and which are simply consequences thereof, but it is thought that by altering the levels of different communication molecules in the brain many of the deleterious age-related changes could be kept at bay. It is possible that the increased inflammation and impaired protein homeostasis that come with age create the right conditions for neurodegenerative disease to develop. Which one an individual eventually succumbs to in their lifetime then could be due to additional genetic or lifestyle risk factors.

1.7 Microglia

Microglia are a type of glial cell that makes up about 10% of total brain tissue and during homeostasis maintain an even spread among all brain areas. They were first identified by Santiago Ramón y Cajal in 1913 and described and named by Pío Del Río-Hortega (Pérez-Cerdá et al. 2015). Like all glial cells their role in the brain is supportive. Microglia are mononuclear phagocytic cells and are the brain parenchyma's only resident macrophage (Li and Barres 2017; Prinz and Priller 2014). Initial bone marrow experiments in mice suggested that microglia had a bone marrow origin (Hickey and Kimura 1988). However, DNA labelling studies later on pointed to microglia being a stable and persistent population (Lawson et al. 1992). That notion was later challenged by a new set of studies on bone marrow chimera mice that showed continuous replacement of microglia by bone marrow derived precursors (Priller et al. 2001). It was later shown that the radiation used to generate mouse chimeras caused brain inflammation which was the cause for the migration of bone marrow precursors (Mildner et al. 2007). It is now accepted that microglia are derived from yolk-sac macrophage precursors and are genetically different from blood mononuclear cells (Prinz and Priller 2014).

Microglia play an important role in development of the CNS and also CNS homeostasis in health and disease. It is unclear why microglia have a unique origin or why they reside only in the CNS. They are not the only immune cell in the brain; perivascular, meningeal and choroid plexus macrophages also reside at the CNS interface (Goldmann et al. 2016). Additionally, peripheral macrophages are capable of migrating through the blood-brain barrier. However, unlike other macrophages microglia haven't been found to be continuously renewed from myeloid progenitor blood cells and instead they increase in number through cell division (Askew et al. 2017). As the microglial population is predefined early in development it is not surprising that they are vulnerable to physiological disturbances such as ageing that can contribute to the development of psychiatric and neurodegenerative diseases.

Until recently microglia were viewed under the misconception that they were static bystanders and only acted in conditions of injury or disease. Now it has been shown that microglia constantly interact with different CNS components and have a central role in the maintenance of brain homeostasis. In development microglia migrate from the yolk sac to the CNS at approximately the same time neurons are formed. Thus, microglia participate in many important events in the developing CNS such as neurogenesis, apoptosis, synaptic pruning and modelling of neural networks (Pont-Lezica et al. 2014; Hoshiko et al. 2012; Paolicelli et al. 2011; Prinz and Priller 2014).

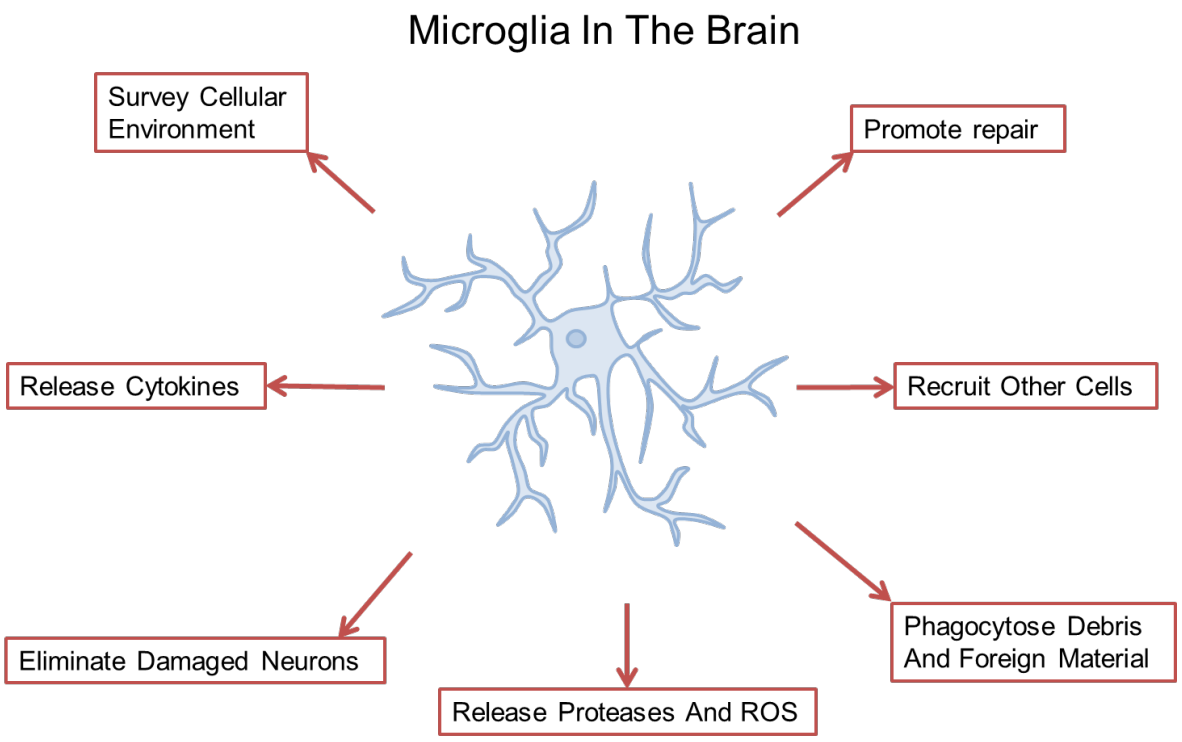

Figure 1.6 Microglia in the brain. Healthy microglial cells play a myriad of supportive roles in the brain including sensing, intercellular communication, promotion of inflammation, degradation and repair.

In the adult organism microglia maintain that multifunctionality and are capable of quickly adapting to changing conditions to adopt a variety of states (Figure 1.6). In the healthy brain microglia maintain a quiescent “resting” phenotype which is maintained by soluble factors secreted by healthy neurons and increased levels of microRNA-124 (Conrad and Dittel 2011). Resting microglia can be recognised by their ramified morphology. Ramified microglia constantly screen the brain with their highly motile processes for signs of damage-associated molecular pattern molecules (DAMPs) such as ATP or calcium release (Nimmerjahn et al. 2005). The most morphologically adaptable and motile cell in the brain, they have been reported to constantly extend and protract their processes and be able to contact the synapses of neurons and secrete communication molecules to regulate them (Panatier and Robitaille 2012; Hristovska and Pascual 2015).

If signs of damage are detected the microglia migrate to the site of injury and convert to an activated or reactive state (Nimmerjahn et al. 2005). The morphology of microglia is an identifying characteristic of their activation state. Non-phagocytic reactive microglia display a thickening of their branches, upregulation of MHCI/II, secretion of proinflammatory cytokines and ROS (Colton 2009; Rock et al. 2004; Gehrmann et al. 1995). As they progress in their activation they can assume a phagocytic state which is characterised by a large ameboid shape and in addition to the aforementioned inflammatory signals they gain the ability to phagocytose material and display it for T-cells (Gehrmann et al. 1995; Rock et al. 2004; Aloisi 2001).

It is thought that depending on the signals they receive microglia can polarise to states similar to M1 and M2 in macrophages where M1 is pro-inflammatory and M2 is phagocytic and aids in tissue regeneration. However, the M1/M2 model fails to account for the complexity of the brain environment and the variety of signals microglia are exposed to by different cell types. Sequencing of microglial transcriptomes in resting and reactive states has found their reactive signature is diverse and difficult to characterise (Hirbec et al. 2018; Wes et al. 2016). Some have argued that the M1/M2 distinction should be done away with altogether as it was developed through *in vitro* experiments in a simplified environment that cannot be replicated *in vivo* (Ransohoff 2016). In the human brain microglia are found to adopt intermediate activated phenotypes demonstrating the complex role these myeloid cells are expected to play in the brain. That has led to the agreement that describing activation states in macrophages including microglia is best done by referring to the stimulating molecule that produced the reactive state (Wes et al. 2016). Just as it has been proven difficult to distinguish specific activation states in microglia on the molecular level, so it has been hard to identify the specific morphology of those cells in live tissue. Historically, most observations of microglia have focused on diseased states and on fixed tissue. As mentioned above microglia are highly reactive cells that can change both their morphology and their molecular signature with changing conditions. With the emergence of more accurate techniques to image live tissue such as two-photon laser scanning microscopy (2P-LSM) we are beginning to get a better understanding of microglial motility and morphological spectrum in physiological conditions (Stopper et al. 2018; Hristovska and Pascual 2015).

Microglial priming is a process driven by changes in the molecular environment including exposure to molecules that drive proliferation (Perry and Holmes 2014). It consists of the increased reactivity of microglial cells upon stimulation. It has been reported in aged mice upon stimulation with IL-1β and IL-12 (Lee et al. 2013). Two types of priming have been reported, classic which has been linked to exposure to IFN-γ and is neurotoxic and alternative – linked to exposure to IL-4 and IL-13 suggested to be neuroprotective (Hickman et al. 2013). Toll-like receptors 2,3 and 4 have been shown to be essential for the classical priming process (Facci et al. 2015). The transcriptional signature of microglial priming seems to be dependent on the High mobility group box 1 (HMGB1) and inhibiting it prevents microglia from entering a primed state (Holtman et al. 2015; Fonken et al. 2016).

1.7.1 Microglial aging

Evidence of microglial aging was first identified in the brains of aged individuals by immunohistochemistry. They were found to be widespread and were characterised by a dystrophic morphology that included process deramification, shortening, gnarling and beading, the formation of spheroids and cytoplasmic fragmentation. It was this type of dystrophic microglia that were also found to store iron through elevated levels of ferritin (Lopes et al. 2008). The accumulation of iron in microglia with age is an interesting phenomenon as microglia are not the main iron-storing cell type in the brain and iron may play a major role in the age-related microglial dystrophy.

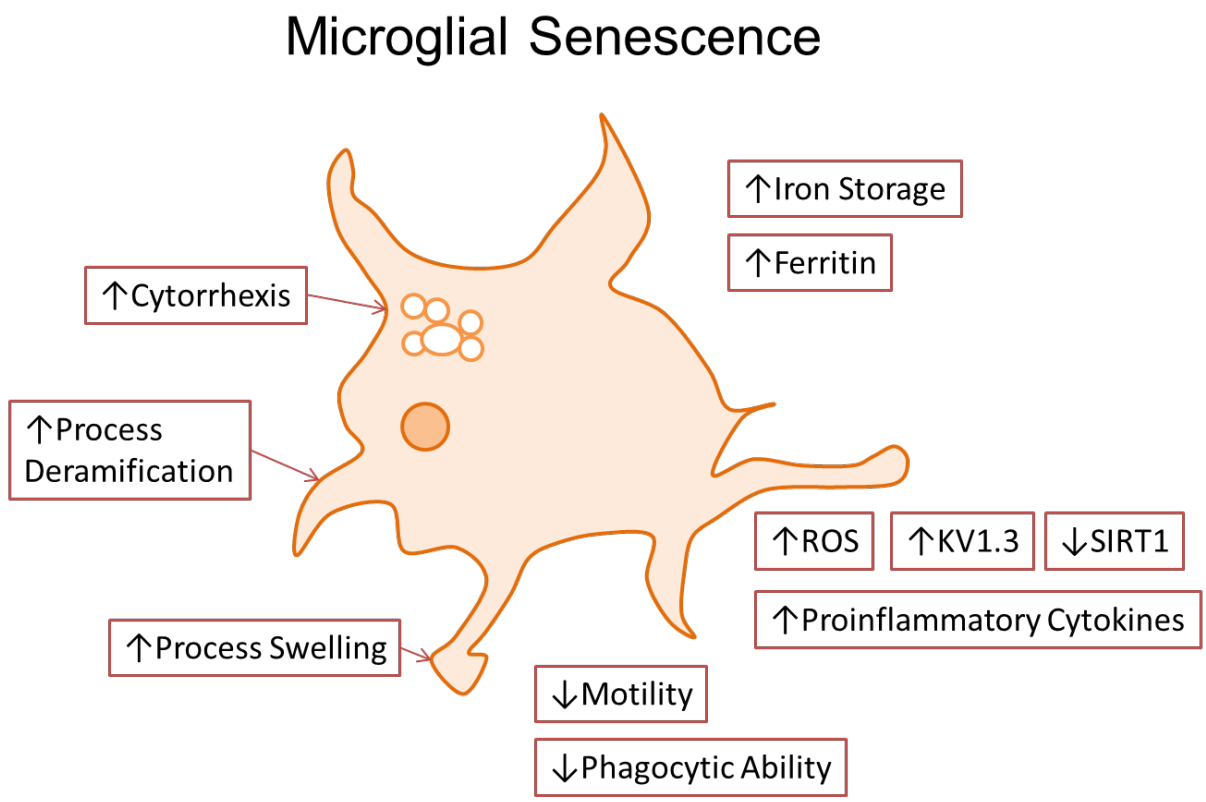

Figure 1.7 Characteristics of microglial aging. When microglia age they lose process ramification, develop process abnormalities and exhibit cytoplasmic fragmentation. They show increased iron storage and ferritin expression. Their increased release of neurotoxic substances and reduced ability to phagocytose debris and toxic protein aggregates leaves neurons vulnerable.

Aging microglia have also been found to have an altered resting phenotype with less dendritic branching and reduced process motility (Figure 1.7). When confronted with injury they exhibit lower migration rates and have a more sustained inflammatory response in reaction to damage (Damani et al. 2011). This characteristic increase in low-grade inflammation is typical of the aging brain with higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines detected in aged brains. Microglia have been found to be very long-lived cells in a study that monitored them over the mouse lifespan (Weinberg 2008). This puts microglia as ideal candidates for chronic senescence and explains how microglial senescence can potentially have a major effect in ND. As microglia are the main immune cells in the brain the way they affect inflammation in the brain has been studied extensively. Aged microglia have been found to not only change their cytokine signature to a pro-inflammatory one but also exhibit reduced phagocytosis and increased ROS production. These changes result in microglia not only failing to maintain neuronal health but also impairing it thus contributing to the possible development of neurodegenerative diseases. The senescence associated secretory phenotype (SASP) is a newer method of characterising aged microglia (Streit et al. 2014). The criteria for SASP differ from cell type to cell type, but microglia have been found to undergo SASP-congruent increases in TNFα, IL1β, IL-6 and IL-8 (Sierra et al. 2007). Interestingly, one of the criteria for developing SASP in a cell type is DNA damage (Coppé et al. 2010). DNA damage has been found to increase in aged microglia, particularly in

mitochondria (von Bernhardi et al. 2015; Hayashi et al. 2008). Telomere shortening is also found in human and rat aged microglia, especially in association with dystrophy (Flanary and Streit 2004). It is likely that a SASP signature will be established for aging microglia in the near future.

The genomic signature of aged microglia has been of great interest since the advent of next generation sequencing technologies. Unfortunately attempts to characterise them have yielded varied and often conflicting results with no clear and consistent specific genetic or protein markers for aged microglia emerging (Crotti and Ransohoff 2016; Spittau 2017; Olah et al. 2018; Wes et al. 2016). The aged microglia in the mouse retina exhibited significant changes in genes controlling inflammation including the NFkB signalling pathway and upregulated complement genes C3 and complement factor B (Cfb) (Ma et al. 2013). A study on the microglial sensome of aged mice reported a shift in microglia to a more neuroprotective phenotype with age with a particular decrease in genes related to sensing endogenous ligands but not phagocytosis or exogenous sensing (Hickman et al. 2013). Those two studies suggested that microglial aging is associated with neuroprotection. In contrast, another study on aged mouse microglia gene expression reported decreased expression in cytoskeletal reorganization suggesting aged microglia are less motile. They also observed increased expression of cytokine genes suggesting that microglia have a reduced ability to migrate to sites in injury and stimulate inflammation, suggesting they play a less neuroprotective role (Orre et al. 2014). A recent gene expression meta-analysis reported that aged microglia display a different expression signature from LPS or IL-4 stimulated microglia, suggesting they don't exhibit a classic activation state but do overlap with primed microglia which are also neurotoxic (Holtman et al. 2015). The few datasets currently published on human microglia aging transcriptome point in the direction that mouse and human microglia may age differently (Galatro et al. 2017; Olah et al. 2018). Olah et. al. (2018) detected significant changes in more than 2000 genes in aged microglia. The pathways that were upregulated were involved in amyloid fibril formation while the TGF-β signalling pathway was downregulated thus suggesting a more pro-inflammatory phenotype for aged microglia. Other upregulated genes were linked to SASP, cytokine signalling, DNA methylation and maintenance among others. That study also validated their expression data with proteomic data as well, finding that many proteins from the amyloid fibril formation gene set were still upregulated in aged microglia. The changes detected in the Galatro et al. (2017) study involved reduced expression of genes related to the actin cytoskeleton and cell surface sensor receptors and alterations in immune response genes. Comparison of this dataset with an existing mouse dataset found very limited overlap in gene expression changes between the two organisms. It is possible that this is due to isolation methods of the cells that may alter their gene expression, but it could also be due to microglia exhibiting different aging signatures in different parts of the brain (Grabert et al. 2016). The differences detected between human and mouse datasets could potentially be explained by the vast disparity in the lifespans of the organisms and of the microglia themselves as a consequence but also by the environments that humans are exposed to that laboratory mice never would. It seems that gene expression studies can reproducibly detect changes in larger networks in aged microglia such as inflammatory response, cytoskeletal remodelling, and ligand sensing; but it's difficult to identify specific biomarkers for identifying aged microglia just from expression data (Holtman et al. 2015). Some proteins related to microglial aging

have been identified in a number of studies that directly investigate the pathways affected by aging.

SIRT1 is a protein related to aging (see above) that has been the object of some interest as its levels decline in many senescent cells. SIRT1 levels were also found to decline in aging microglia both in aged human brain samples and in mouse models (Olah et al. 2018; Cho et al. 2015). Decline in SIRT1 is thought to contribute to age-related neurodegeneration by stimulating inflammatory pathways in microglia through the activation of IL-1β, thus contributing to the SASP phenotype (Cho et al. 2015)

KV1.3 is a potassium channel found in microglia also thought to control inflammatory response (Moussaud et al. 2009). KV1.3 is involved in the secretion of cytokines that are part of the SASP signature of aging microglia (Charolidi et al. 2015). Increased levels of KV1.3 have been detected in aging microglia and recently KV1.3 has been identified as a major controller for pro-inflammatory genes in disease associated microglia in AD models where KV1.3 has also been found to be upregulated (Rangaraju et al. 2018). Additionally, KV1.3 inhibition has recently been suggested as a potential therapy targeting microglia in AD (Maezawa et al. 2018).

The triggering receptor expressed on myeloid cells 2 (TREM2) signalling pathway is another interesting example. TREM2 is a central microglial cell surface receptor that is thought to regulate microglial survival, inflammatory signalling, migration and phagocytosis. Changes in TREM2 expression and signalling in aging can lead to increased inflammation and reduced phagocytosis (Forabosco et al. 2013). The release of a soluble form of TREM2 (sTREM2) further complicates the role for this receptor in aging. The understanding of the function of TREM2 in the healthy brain and in neurodegeneration is still limited (Mecca et al. 2018). It is important to identify the reason why all these signalling pathways change and play a role in microglial aging.

1.7.2 Models of aged microglia

The study of aging microglia is important not only in terms of understanding their phenotype and gene expression, but also in understanding what their role is in both the aging of the CNS and in ND. Thus, having models of aging microglia that can be used to study their effect on neurons is extremely important. Obviously, aging microglia can be found in aged animals and some studies have utilised them in the study of senescence. (Sierra et al. 2007; Griffin et al. 2006; Letiembre et al. 2007; Stichel and Luebbert 2007; Perry et al. 1993; Godbout et al. 2005) Unfortunately, using aged animals for experiments can be exceedingly expensive and time consuming, thus making them very impractical. Additionally, despite some reported methods isolating and maintaining aged microglia in culture has by and large proven to be very difficult (Von Bernhardi et al. 2011). Some have made the case for the importance of studying age-related diseases in aged models being important enough to be worth the extra costs. However, currently it is just as difficult to obtain aged animals as ever (Johnson 2015). Transgenic models of accelerated aging are another potential avenue for observing microglial aging. The Ercc1(-/Δ) transgenic mouse model displays accelerated aging through DNA-repair deficiency (Schermer et al. 2013). This model has shown age-related changes in microglia(Raj et al. 2014). Another model of accelerated aging is the mTerc$^{-/-}$ mouse that exhibits telomere shortening whose

microglia exhibit some signs of senescence and priming (Raj et al. 2015). An alternative to a full animal model can be cell culture models of aged microglia. One study has reported that microglia isolated from neonatal mice start resembling aged microglia with time in culture, including reduced phagocytic ability, reduced motility, reduced autophagy, and changes in microRNAs and SA-β-galactosidase activity (Caldeira et al. 2014). However, these cells didn't exhibit the SASP expected from aging microglia. Additionally, at 16 days this model is very short lived, thus again making it impractical for the study of neurodegeneration. Another method to induce age-like changes in microglia has recently been presented by Park et al. (2018). By treating primary rat microglial cultures with the drug dexamethasone (a corticosteroid) they simulate chronic stress inflicted by steroid hormones that microglia experience over time with aging. This model exhibited increased SA-β-galactosidase activity, increased expression of tumour suppressor genes and dysfunctional phagocytosis similarly to senescent cells. However, they also showed increased autophagy, decreased expression of inflammatory genes and decreased cytokine release which is unlike aged microglia.

The utility of an easily replicable model of microglial aging is clear as it would enable a major risk factor of neurodegeneration to be included in the study of these diseases (Koellhoffer et al. 2017).

1.7.3 Aged microglia and neurodegeneration

Healthy microglial cells are essential to neuronal survival as they maintain brain homeostasis and fight off infection through a complex signalling system driven by secreted factors. Activated microglia were thought to be the cause of inflammation seen in neurodegenerative disease. However, senescent microglia present both the impaired neuroprotective ability and the low but sustained secretion of molecules that drive inflammation seen in neurodegeneration (Koellhoffer et al. 2017). Dystrophic microglia have been identified in both aged brains and the brains of patients with neurodegenerative disease. They have been found near sites of tau pathology and amyloid plaques of AD brains and near Lewy bodies in dementia with Lewy bodies brains (Lopes et al. 2008; Streit et al. 2004; Streit et al. 2009; Streit and Xue 2016). In neurodegenerative disease microglia seem to adopt an intermediate phenotype where they present both markers of M1 and M2 activation. Chronically activated microglia have been found in the brains of patients with a multitude of neurodegenerative diseases such as AD, PD, ALS and prion disease. It isn't known why microglia adopt this phenotype. A comparison of microglial markers between individuals with AD and high-pathology but cognitively normal controls showed that microglial activation is increased in AD, suggesting that microglial activation is not simply a reaction to AD pathology such as β-amyloid deposition (Hopperton et al. 2018). It is possible microglia are pushed towards a more proinflammatory phenotype through age-related changes.

Mutations in genes related to microglial function have been linked to neurodegenerative disease risk. For example, a rare variant of the *TREM2* gene leads to an increase in the risk for developing AD 3-4 times and more severe pathology. In the context of both aging and neurodegeneration TREM2 is thought to interact with APOE activate pathways linked to microglial activation, survival and phagocytosis (Krasemann et al. 2017). Loss of

function mutations in TREM2 also cause a rare deadly disease (Nasu-Hakola Disease) that presents with neurodegenerative symptoms and bone cysts (Yeh et al. 2017). The expression of soluble TREM2 by aged microglia of a mouse AD model was also found to rise with both amyloid load and markers of microglial activation over time, suggesting that sTREM2 also plays a role in the neurodegenerative process (Brendel et al. 2017). Leucine rich repeat kinase 2 (LRRK2) is a protein expressed highly in microglia that is implicated in both microglial activation and lysosomal degradation. Mutations in LRRK2 are the most common in both familial and sporadic PD. They are thought result in increased proinflammatory signalling and possibly preventing microglial cells from degrading protein aggregates (Schapansky et al. 2015; Gillardon et al. 2012). CSF1R is a cell surface receptor for the cytokine CSF1 and in the brain it is expressed predominantly in microglia and is a mediator for microglial proliferation and differentiation. CSF1R loss of function mutations have been shown to cause hereditary diffuse leukoencephalopathy with spheroids (HDLS), a neurodegenerative disease that has symptoms of dementia and parkinsonism among others (Rademakers et al. 2012). CD33 is another microglial cell surface receptor that has both found to be upregulated in AD and has a rare variant that confers increased risk of developing AD. CD33 acts to reduce microglial proliferation and phagocytosis and thus exacerbates AD pathology (Griciuc et al. 2013; Malik et al. 2013). These examples show that microglial function is essential to maintaining neuronal homeostasis and health and thus any disruption in microglial function can result in neurodegeneration.

As immune cells, microglia have been found to react to the presence of misfolded proteins in both AD and PD. Microglia are well known to be activated by the presence of β-amyloid and to cluster around sites of amyloid plaques. As the main protein that aggregates in PD, α-synuclein can also interact with microglia. Microglia have been shown to react to aggregated α-synuclein but not to monomeric through TLR receptor activation (Béraud et al. 2013; Fellner et al. 2013). The TLR2 receptor has been shown to be activated by the most likely toxic form of α-synuclein aggregates – β-rich oligomers(Kim et al. 2013). Additionally, microglia can also become activated through the detection of neurons under stress. Activated microglia secrete IL1β and TNFα that have been found to contribute to neuronal demise through the recruitment of neutrophils and chemokines from blood. Through the secretion of proinflammatory signals and ROS activated microglia can lead to the retrograde apoptosis of neurons that are connected to the degenerating neurons thus spreading the pathology. Therefore, through inappropriate action microglia are capable of perpetuating neurodegeneration in a feedback loop of inflammatory signalling (Perry et al. 2010).

As phagocytes, one of the roles of microglia is to clear any cellular debris and protein aggregates that they encounter. Therefore, one of the functions of microglia is degradation of β-amyloid. However, aging microglia lose their ability to clear β-amyloid effectively from the extracellular space. In mouse models aged microglia have been found to have reduced expression of β-amyloid degrading enzymes and reduced phagocytosis (S. E. Hickman et al. 2008). It is thought that in AD microglial function is impaired by the mere presence of β-amyloid aggregates, thus leading to a self-perpetuating cycle of increased β-amyloid accumulation and further damage. A systematic review of microglial markers in AD found that most studies observed increases in markers related to microglial activation, but no significant difference in overall microglial markers.

They also found no difference in overall cell counts suggesting that microglial number does not increase in AD, but microglial levels of activation do (Hopperton et al. 2018).

Inflammation and ineffective protein clearance are strongly linked in neurodegeneration. It has been shown that microglia play a major role in the pathology of Alzheimer's disease and Parkinson's disease by driving inflammation in the brain (Subramaniam and Federoff 2017). In fact, in a mouse model, eliminating microglia didn't affect amyloid deposition but prevented neuronal loss and degeneration and also resulted in improved cognition (Spangenberg et al. 2016; Dagher et al. 2015). Reducing inflammation by the use of NSAIDs is also linked to a lower risk of developing PD (Gagne and Power 2010). In an organotypic cell culture model of β-amyloid accumulation in the aged brain, old microglia's ability to clear β-amyloid was rescued by the presence or the conditioned medium of young microglia, suggesting that the effects of microglial aging on amyloid deposition can be reversed (Daria et al. 2017).

Microglia's ability to clear aggregated proteins may be linked to their age or state of activation. Aged microglia in models of β-amyloid load that exhibited a lower phagocytic ability were also found to express the cytokines TNFα and IL-1β, unlike microglia that did phagocytose β-amyloid (Hickman et al. 2008). The expression of inflammatory cytokines is thought to also drive further microglial damage and perpetuate the AD pathology. Microglia have also been shown to be able to internalise and degrade extracellular α-synuclein aggregates in a cell culture study, an activity that is apparently controlled by the activation state of the cells as it was reduced by activation with lipopolysaccharide (LPS) (Lee et al. 2008).

The role of iron-rich microglia in neurodegenerative disease cannot be ignored. Ferritin-positive dystrophic microglia have also been found associated with amyloid plaques and neurofibrillary tangles (Streit et al. 2014). Iron also accumulates in AD brains in the hippocampus and in particular in the amyloid plaques of AD patients (Raven et al. 2013; Smith et al. 1997). It is possible that iron together with other metal ions plays a role in the toxicity of β-amyloid oligomers (Tabner et al. 2011). Iron appears to also accumulate excessively in regions affected by Parkinson's disease such as the *substantia nigra* and is associated with Lewy bodies where dystrophic ferritin positive microglia have been identified (H. Xu et al. 2018). Disruption in iron homeostasis has also been linked with a higher secretion of proinflammatory cytokines in microglia *in vitro* (Wang et al. 2013). Furthermore, short-term iron exposure has been found to induce rat primary microglia to potentiate neurotoxicity (Zhang et al. 2013). High lifetime exposure to iron has been linked to an increased risk of developing PD. Neuromelanin, a protein that stores iron in neurons can be phagocytosed by microglia attracted to degenerating neurons, thus increasing the iron load of those cells. Interestingly, it has been demonstrated that neuromelanin phagocytosis can induce increased release of proinflammatory cytokines and ROS, thus driving inflammatory processes that can contribute to neuronal degeneration further (Rathnasamy et al. 2013). It is possible that iron accumulation in microglia could be a protective mechanism from iron toxicity in the brain which then can proceed to damage the microglia themselves and induce the accelerated aging signature seen in neurodegeneration (Krabbe et al. 2013).

1.8 Hypothesis, Aims and Objectives

1.8.1 Hypothesis

The main hypothesis of this thesis is that microglia acquire a pathological phenotype with age that is linked to the accumulation of iron and that this phenotype plays a role in the processes that initiate neurodegenerative disease. It is further hypothesized that this phenotype can be modelled by forcing microglia to take up iron and can be used to incorporate an aging aspect into the study of neurodegenerative disease.

1.8.2 Aims and objectives

There is a clear gap in knowledge related to how aging in the brain affects the pathogenesis of neurodegenerative disease. This thesis aims to provide a link between factors affected in ageing in the brain and the processing and toxicity of proteins heavily implicated in neurodegeneration. Even though it is now known that microglia are linked to the pathology of AD and PD and that their phenotype changes with age, not many studies have incorporated microglial aging in the study of those diseases.

1. The first objective is to investigate whether supplementing human and mouse microglial cell lines and primary mouse microglia with iron changes them to an aged phenotype and to characterise it for iron storage, expression of cytokines, proteins and other molecules linked to aged microglia.
2. The second objective is to find out if a human iron-fed microglia cell line can be applied to the study of β-amyloid aggregation and to show whether the soluble factors released by these model aged microglia in their conditioned medium can change the detected levels of β-amyloid in a neuronal cell line. If it is found that β-amyloid levels are influenced by conditioned medium the mechanism will be determined. This will be pursued by analysing whether break down or release of β-amyloid is affected. The level of activity of secretases and changes in the levels of proteases secreted by microglia such as IDE or neprilysin will be analysed.
3. The next objective is to determine whether conditioned medium from iron-fed mouse primary and cell line microglia can influence both α-synuclein expression in neuronal cells and the formation of toxic α-synuclein aggregates. If this is found to be the case the molecule present in conditioned medium that caused the changes will be identified. A likely candidate for this would be inflammatory cytokines. If a particular cytokine or combination of cytokines is found to elicit those changes, the pathway through which those act will be investigated through pharmacological inhibitors.
4. The final objective will be to investigate the downstream effects of the aged microglial model on α-synuclein. Overexpressing α-synuclein but not β-synuclein was found to make a neuronal cell line more vulnerable to α-synuclein oligomer toxicity. This process could be mediated through increased FOXO3a expression in the α-synuclein overexpressing cell line. If that is the case, it will be tested if FOXO3a levels increase after treating the neuronal cell line with Fe^{2+} as a possible source of ROS. If iron treatment is found to elevate FOXO3a levels it will be tested if the overexpression of another known ferrireductase (STEAP3) and another synuclein protein (β-synuclein) can replicate the effect of α-synuclein on FOXO3a expression. Finally, the effect of β-synuclein overexpression on iron and ROS levels in neuronal cells will be investigated as a potential explanation for its observed protective ability.

2. Altered Processing of ß-amyloid in SH-SY5Y cells induced by Model Senescent Microglia

2.1 Introductory commentary

As mentioned in the Introduction the lack of models of microglial aging is detrimental to the study of neurodegeneration. A good model of senescent microglia would ideally be easy to induce, replicate and would replicate the changes seen in microglial aging.

Papers published by the Streit group show that the accumulation of iron is an interesting quality of aging microglia. (Streit et al. 2014; Lopes et al. 2008; Streit et al. 2004). This finding inspired us to investigate how the accumulation of iron by microglia alters their phenotype. We investigated this question in cell culture as it allows us to manipulate and measure the cellular environment and phenotype of microglia with more precision. Microglial cells in culture have been shown to have a high resistance to iron toxicity in the medium (Bishop et al. 2011). Preliminary data generated by our laboratory showed that simply incubating microglial cells with excess iron causes them to change morphology. This resulted in the hypothesis that culturing microglia with iron results in a change in the factors that they secrete to communicate with neurons.

The work presented in the next chapter aims to characterise iron-fed microglia as a cell culture model of aged human microglia. A human microglia cell line was forced to adopt an aged phenotype by incubation with ferric ammonium citrate for two weeks. The second aim of the paper is to show that this model can be used in the study of neurodegenerative disease by investigating the effect of the microglial conditioned medium on β-amyloid released by SHSY5Y neuronal cells. The work focuses on find out to what extent this model matches the phenotype of aged microglia described in the literature and on understanding the mechanism behind the observed alteration in β-amyloid processing by measuring key proteins and pathways. The SV-40 microglial cell line chosen in this study was derived from human primary microglial cells and shown to express the microglial markers TREM2 and Iba1 (Patel et al. 2016). They were chosen due to the difficulty in obtaining primary human microglia but also because this cell line allowed the generation of sustainably large numbers of microglia that were necessary for generating the data. Conditioned medium from the microglia was used when assessing their effect on neuronal cells as this reduces the number of variables that need to be measured down to molecules secreted by the microglia.

2.2 Statement of contribution

This declaration concerns the article entitled:									
Altered Processing of Beta-amyloid in SH-SY5Y cells induced by Model Senescent Microglia.									
Publication status (tick one)									
draft manuscript		**Submitted**		**In review**		**Accepted**		**Published**	X
Publication details (reference)	Angelova, D & Brown, D 2018, 'Altered Processing of Beta-amyloid in SH-SY5Y cells induced by Model Senescent Microglia.' ACS Chemical Neuroscience. DOI: 10.1021/acschemneuro.8b00334								
Candidate's contribution to the paper (detailed, and also given as a percentage).	The candidate contributed to/ considerably contributed to/predominantly executed the… Formulation of ideas: Identification of interesting targets for analysis once the basic concept had been proven. (60%) Design of methodology: Modification of cell culture, western blot and kit assay protocols to obtain quality data. (75%) Experimental work: All experimental work was performed. Generated the data presented in all figures of the paper. (100%). Presentation of data in journal format: Preparation of figures and tables, proof reading of the manuscript. (50%)								
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.								
Signed	Dafina Angelova					**Date**	18.09.18		

2.3 Permissions

Title: Altered Processing of β-Amyloid in SH-SY5Y Cells Induced by Model Senescent Microglia

Author: Dafina M. Angelova, David R. Brown

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Altered Processing of β-Amyloid in SH-SY5Y Cells Induced by Model Senescent Microglia

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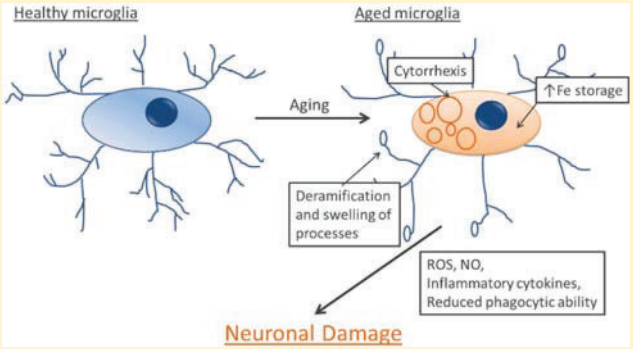
Supporting Information

ABSTRACT: The single greatest risk factor for neurodegenerative diseases is aging. Aging of cells such as microglia in the nervous system has an impact not only on the ability of those cells to function but also on cells they interact with. We have developed a model microglia system that recapitulates the dystrophic/senescent phenotype, and we have combined this with the study of β-amyloid processing. The model is based on the observation that aged microglia have increased iron content. By overloading a human microglial cell line with iron, we were able to change the secretory profile of the microglia. When combining these senescent microglia with SH-SY5Y cells, we noted an increase in extracellular β-amyloid. The increased levels of β-amyloid were due to a decrease in the release of insulin-degrading enzyme by the model senescent microglia. Further analysis revealed that the senescent microglia showed both decreased autophagy and increased ER stress. These studies demonstrate the potential impact of an aging microglial population in terms of β-amyloid produced by neurons, which could play a causal role in diseases like Alzheimer's disease. Our results also further develop the potential utility of an *in vitro* model of senescent microglia for the study of brain aging and neurodegenerative disease.



KEYWORDS: *β-amyloid, APP, microglia, ER stress, autophagy, insulin degrading enzyme*

INTRODUCTION

There is overwhelming evidence for the importance of aging to the etiology of neurodegenerative diseases.[1] Conditions like Alzheimer's disease (AD) increase in frequency as we age.[2] There are numerous diseases, including inherited forms of AD and prion diseases, in which dominant inherited mutations lead to neurodegeneration but only when the carrier passes a certain age.[3,4] Clearly understanding how the brain changes with age and how such changes result in the development of diseases involving neuronal dysfunction and loss is essential for understanding neurodegeneration. Therefore, it is quite surprising that very few studies incorporate any aspect of the aging brain in their models. There is an inherent difficulty in including an aspect of aging in the development of a model of neurodegeneration because of the time requirement; for example, animal models would have to utilize animals that are toward the end of their life span.[5] *In vitro* models have even greater hurdles because such models are inherently short-term.[6,7] Therefore, a potential alternative is to develop a method to induce a phenotypic change equivalent to that seen in cells from an aged brain.

Microglia are important cells in the brain that maintain neuronal well-being.[8] There is a plethora of information suggesting that microglia may participate in changes in the brain associated with neurodegenerative diseases.[9−15] These changes mostly relate to an altered secretory profile wherein the molecules released either result in reduced protection of neurons or an increase in proinflammatory or toxic molecules such as cytokines or reactive oxygen species.[16] Microglia change phenotype in this way with age, and aged microglia are frequently described as dystrophic.[17] Microglia may develop a senescent-associated secretory phenotype (SASP).[18−20] While SASP is mostly associated with a study of molecular changes in cells, the dystrophic phenotype has largely been assigned on the basis of morphological changes. While both phenotypes are age associated, they have been rarely used in conjunction to describe aged microglia. Understanding how microglia could enter such a phenotypic state and the potential of microglia to alter neuronal activity as a result is of considerable importance. Phenotypically, dystrophic microglia express increased levels of the iron storage protein ferritin,[21,22] which is directly related to the increased levels of iron stored by them.[23] Increased iron levels in the brain are associated with both aging and patients with a variety of neurodegenerative diseases including AD and Parkinson's disease.[24−27]

Dystrophic/senescent microglia are present in the brains of patients with AD.[20,28] However, while their presence has been shown, any causative role is unknown. AD is mostly associated

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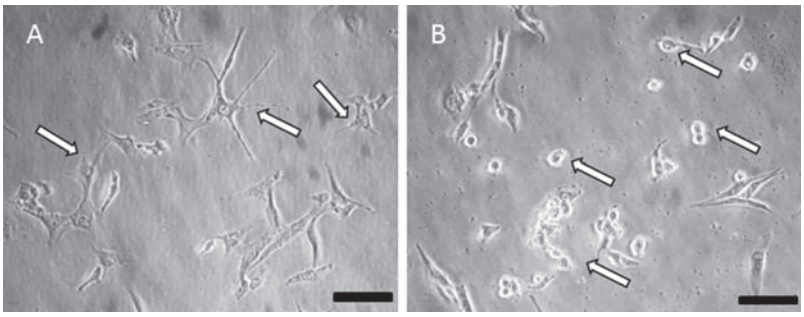

Figure 1. Iron-fed microglia in culture. Photomicrographs of human immortalized microglia in culture. (A) Microglia grown in control conditions showed many fine process (arrows). (B) Microglia grown in 500 μM ferric ammonium citrate (iron-fed) for at least 2 weeks showed a loss of these processes. Arrows indicated more amoeboid appearance. Scale bar = 100 μm.

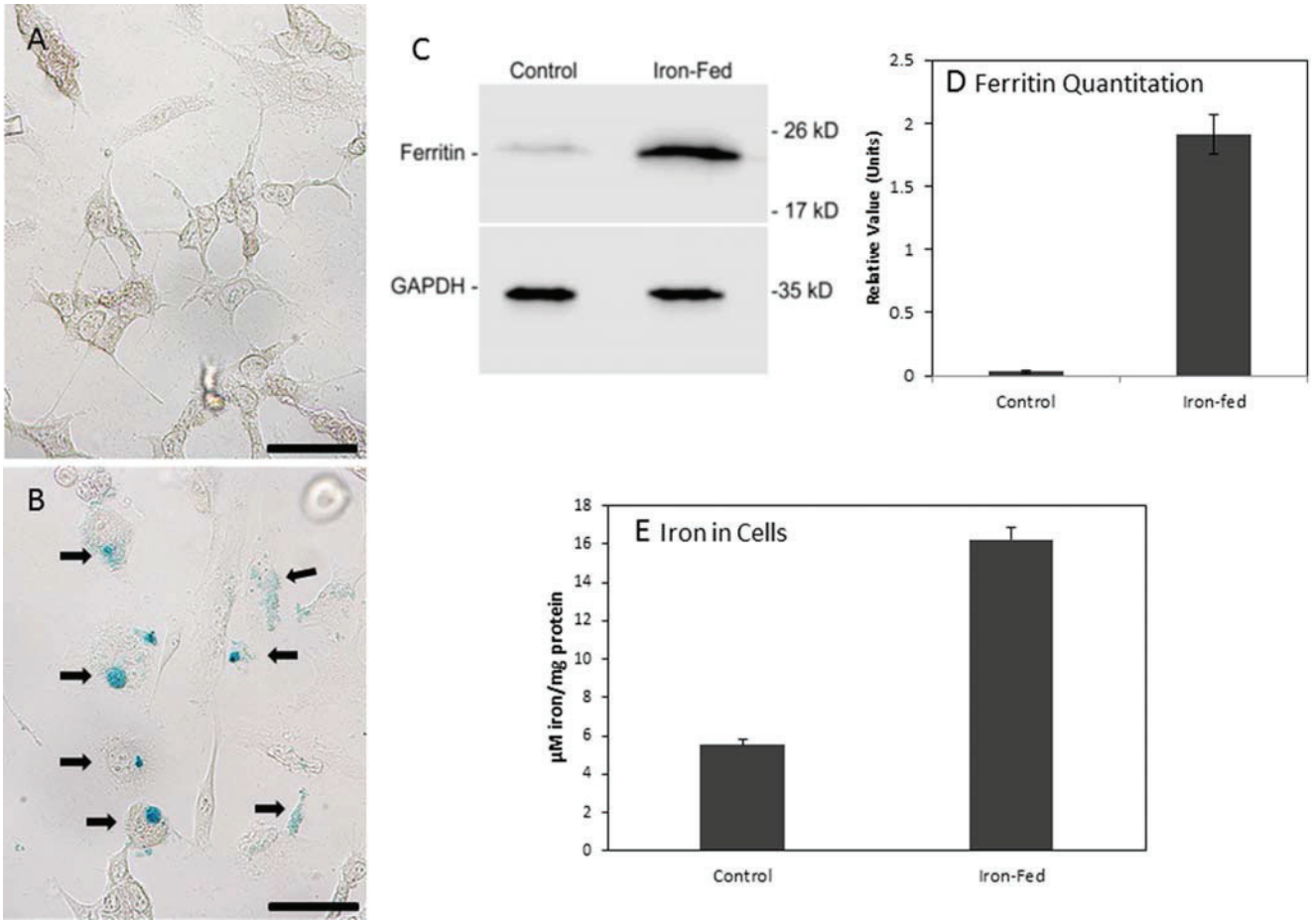

Figure 2. Iron and ferritin in iron-fed microglia. Perl's stain is a traditional stain for the detection of iron deposits in cells. Cultured microglia were stained using Perl's stain. Iron deposits appear blue following the procedure. (A) Control microglia show no obvious staining, while (B) iron-fed microglia (grown in iron for at least 2 weeks) show extensive blue staining in almost all cells (arrows). (C) Western blotting was used to detect ferritin in protein extracts from control and iron-fed microglia. GAPDH was used as loading control. (D) Densitometric analysis of ferritin expression was normalized to GAPDH levels (relative value). Iron-fed microglia show significantly higher levels of ferritin expression ($p < 0.05$) when compared to controls. Shown are the mean and SE of four experiments. (E) The level of iron was detected in extracts from microglia using a commercial kit. The iron-fed microglia showed very high and significantly different ($p < 0.05$) levels of iron when compared to controls. Shown are the mean and SE of four experiments.

with the deposition of protein aggregates, which include β-amyloid in the form of plaques and tau in the form of paired-helical filaments or tangles.[29,30] Microglia secrete enzymes that are able to degrade β-amyloid such as insulin-degrading enzyme (IDE) and neprilysin.[31,32] There have also been reports suggesting neuronal loss in AD may come from activation of microglia as a result of interaction with β-amyloid deposits.[33,34] While there is considerable interest in microglia in terms of the pathology of AD, the impact of microglial senescence on the etiology of the disease is unknown.

In this work, we examine a new model of senescent microglia based on the observation that overloading microglia

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with iron forces them into a senescent-like phenotype. Combining these microglia with a neuronal cell line allowed us to investigate how the change in phenotype alters the generation of β-amyloid. Following treatment of SH-SY5Y cells with conditioned medium from dystrophic microglia, there is an increase in β-amyloid present in the medium due to a decrease in the secretion of IDE. We have linked this change in IDE release to increased ER stress in microglia induced by the iron overload. The results cast light on the possible mechanism by which brain aging causes increased deposition of β-amyloid, which could lead to AD.

RESULTS

Phenotype of Iron-Fed Microglia. We used a human microglia cell line as the basis of our investigation. This line was chosen because it allowed the generation of sufficient cells for experiments and also because it maintained the species match for experiments involving β-amyloid generated by the human SH-SY5Y neuroblastoma cell line. As aged dystrophic microglia show high levels of stored iron, we hypothesized that the dystrophic phenotype might be a consequence of high retention of iron.[22] Iron (particularly Fe(II)) causes damage to macromolecules and increased storage could be sufficient to induce changes seen in the dystrophic phenotype. For this reason, we grew the human microglial cell line in 500 μM ferric ammonium citrate for at least 2 weeks.

In microglial cells grown in medium with high iron (iron-fed), we observed morphological changes similar to dystrophic microglia.[17] Under normal culture conditions (Figure 1A), microglia cells showed a small cell body with multiple projections and frequent branches. In contrast iron-fed microglia (Figure 1B) showed no branches and little to no projections. In many cases, the microglia became amoeboid.

We verified that iron storage had occurred by three methods. First we used Perl's stain to identify iron deposits in the cells. As can be seen in Figure 2, control microglial cells (Figure 2A) had little to no deposits while iron-fed microglia showed large numbers of iron deposits in blue (Figure 2B). This demonstrates that iron-fed microglia store considerably more iron than the untreated control. Similarly, levels of ferritin are considered to reflect the levels of stored iron in cells. We measured the levels of ferritin in control and iron-fed microglia by western blot and immune detection. The levels of ferritin in iron-fed microglia were much higher than in controls (Figure 2C,D). Lastly, we directly measured the levels of total iron in the microglia using a commercial kit. Iron-fed microglia showed considerably higher levels of total iron (Figure 2E).

Treatment of the microglial cell line with high concentrations of iron may have adversely affected their viability in culture. To control for this, we measured their proliferation when compared to controls using a BrdU incorportation assay. Control and iron-fed human microglia were plated at a range of densities, and the incorporation of BrdU was assessed using an ELISA assay (Figure 3). Regardless of plating density, the iron-fed microglia showed no significant difference in BrdU incorporation suggesting that the iron-fed microglia maintained the same proliferation rate as the untreated controls.

Alteration in the secretory profile is an indication of a potential senescent phenotype in cells.[35] Microglia are known to show increased pro-inflammatory cytokine secretion with age.[18] Therefore, we assessed a panel of cytokines associated with pro-inflammatory responses. Conditioned medium from control and iron-fed microglia were collected and normalized

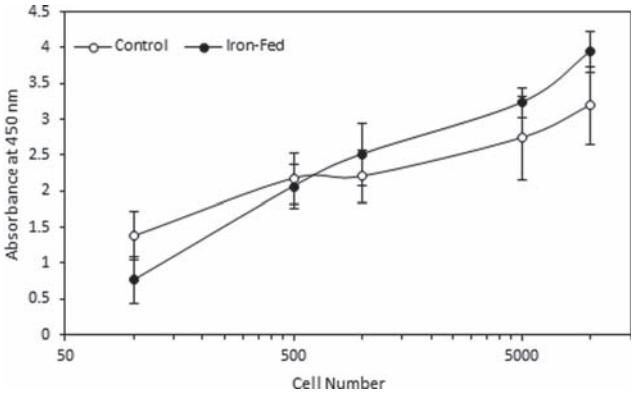

Figure 3. Proliferation of iron-fed microglia. The rate of proliferation of human immortalized microglia was assessed using a BrdU based ELISA kit. Both control and iron-fed microglia were plated onto a 96 well plate at a range of densities and grown overnight. BrdU was then added for a further 16 h before the ELISA assay was used to assess incorporation levels. The level of incorporation was assessed by a colorimetric assay with a read out at 450 nm. Iron-fed microglia showed no significant difference in proliferation at all plating densities ($p > 0.05$). Shown are the mean and SE of four separate experiments.

in relation to the protein content of the microglia used to conditioned the medium. The cytokines measured were IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, and TNF-α (Table 1). Some of the cytokines tested could not be

Table 1. Cytokines Released into Conditioned Medium[a]

cytokine	control	iron-fed
IL-8	47211.41 ± 19621.34	120271.14 ± 22992.87[b]
IL-6	3393.33 ± 1501.62	14181.68 ± 2346.10[b]
IL-1b	35.95 ± 12.72	115.31 ± 23.87[b]
IL-13	1.05 ± 0.16	1.47 ± 0.29
IL-10	0.03 ± 0.00	0.05 ± 0.01
IL-2	0.05 ± 0.04	0.15 ± 0.02
TNFα	0.88 ± 0.47	0.16 ± 0.08
IFNγ	[c]	[c]
IL-4	[c]	[c]
IL-12p70	[c]	[c]

[a]Serum-free conditioned medium was generated from cultured microglia over 24 h, and the levels of cytokines were assessed with the MSD ELISA system. Cytokine concentrations were determined by comparison to a standard curve for each cytokine. Values were the concentration in the medium (ng/mL) divided by the concentration of the protein in the cells that were used to generate the conditioned medium (mg/mL). Shown are the mean (ng/mg) and SE for four experiments. [b]Significant difference between control and iron-fed (p-value <0.05). [c]Not detectible due to the levels detected being below the level of lowest standard on the standard curve for that cytokine.

detected, while others showed no changes. However, a number of the cytokines measured showed significant elevation. These included IL-1β, IL-6, and IL-8. The other measurable cytokines showed no changes (IL-2, IL10, IL13, and TNFα). This selective change in cytokines suggests a change in microglial phenotype similar to that suggested for aged microglia.

We also assessed other reported markers for aged/senescent microglia. It has been suggested that during the aging process microglia show reduced secretion of glutamate.[36] We used a commercial glutamate assay kit to measure glutamate released into culture by control and iron-fed microglia. Figure 4A shows

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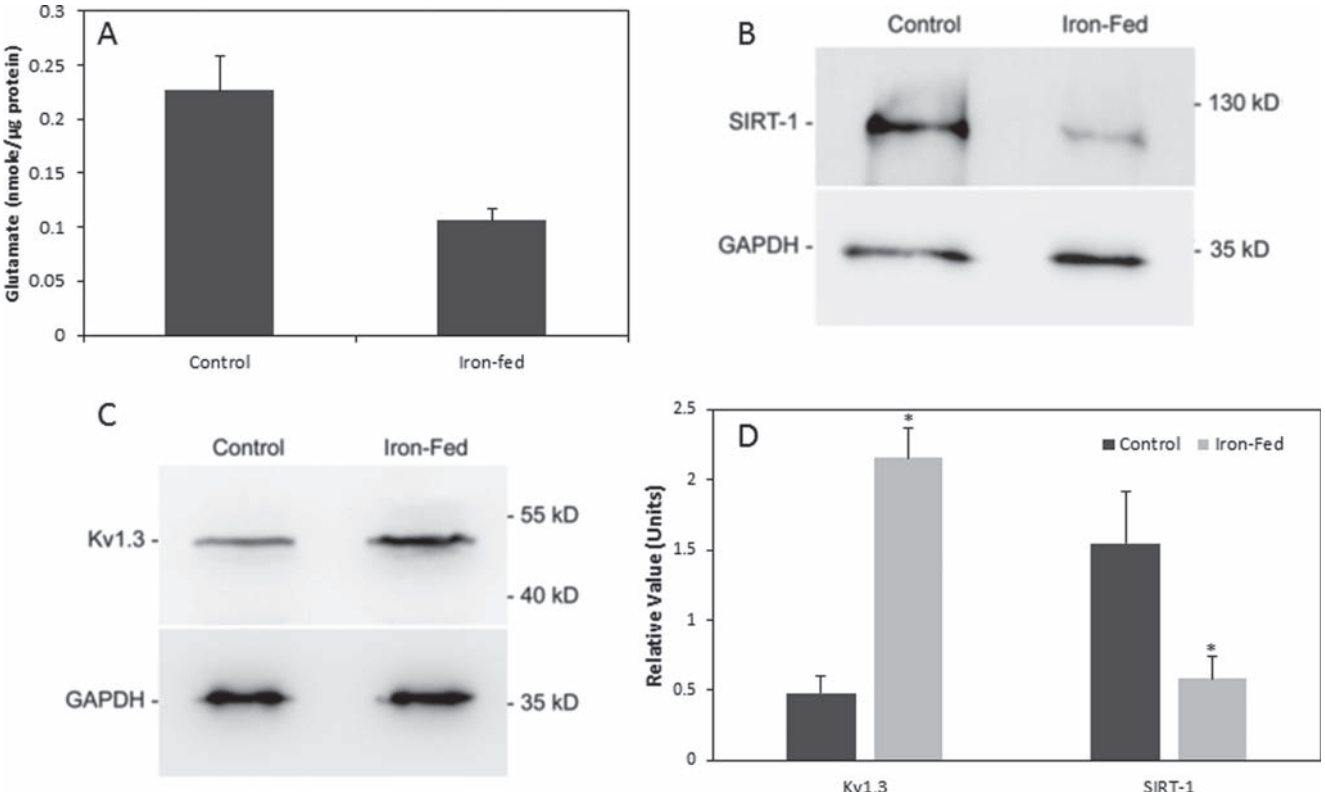

Figure 4. Glutamate release and protein expression iron-fed microglia. (A) The release of glutamate by human immortalized microglia was assessed with a commercial glutamate assay kit. Control and iron-fed microglia plated at equal density were grown in serum free medium for 48 h. The level of glutamate was then determined after collecting the medium. The protein content of the conditioning cells was determined in parallel. Glutamate concentration was assessed relative to the protein content of the cells to account for differences in cell number. Iron-fed microglia released significantly less glutamate than control microglia ($p < 0.05$). (B) Western blotting was used to assess the level of expression of KV1.3 in protein extracts from control and iron-fed microglia. A specific antibody was used to detect KV1.3 and GAPDH. (C) Western blotting was used to assess the level of expression of SIRT-1 in protein extracts from control and iron-fed microglia. A specific antibody was used to detect SIRT-1 and GAPDH. (D) Densitometric analysis of KV1.3 and SIRT-1. Expression was normalized to GAPDH levels (relative value). Iron-fed microglia show significantly higher levels of KV1.3 expression ($p < 0.05$) and significantly lower SIRT-1 expression ($p < 0.05$) when compared to controls. * indicates significant difference. Shown are the mean and SE of four experiments for all parts.

that iron-fed microglia released significantly less glutamate than control microglia. In addition, we measured two known markers for aged microglia. These included KV1.3, a microglial potassium channel, and SIRT-1 (sirtuin-1), a deacetylase. KV1.3 has been shown to be altered in aged mice and to play a role in the release of cytokines.[37,38] SIRT-1 has been shown to be decreased in aged microglia, and this may contribute to cognitive decline and neurodegeneration.[39] We used western blotting and specific antibodies to detect these proteins in extracts from control and iron-fed microglia (Figure 4B–D). Iron-fed microglia showed a significant increase in the level of expression of KV1.3 but a significant decrease in expression of SIRT-1. These results are consistent with the suggestion that iron-fed microglia demonstrate a change in phenotype similar to dystrophic or senescent microglia *in vivo*. We are therefore confident that our iron-fed human microglia represent a robust *in vitro* model of senescent microglia.

Effect of Microglial Conditioned Medium on APP Metabolism in SH-SY5Y Neuronal Cells. Alzheimer's disease is associated with the deposition of abnormal proteins including β-amyloid. The rate of formation of β-amyloid depends upon the metabolism of its precursor, APP, and the relative activity of the two secretase pathways that degrade APP to form either β-amyloid (β-secretase dependent) or P3 (α-secretase dependent). In either pathway, the end result is the release of an APP fragment by the γ-secretase complex. However, the rate of formation of these products could be influenced by the level of expression of APP and the enzymes ADAM10 (α-secretase) and BACE-1 (β-secretase). The model we used to study the levels of these proteins was the human neuroblastoma cell line, SH-SY5Y. Conditioned medium was prepared from control and iron-fed microglia and applied to SH-SY5Y cells for 24 h. After that time, the SH-SY5Y cells were assessed for the expression of these proteins by western blot and immunodetection with specific antibodies. As shown in Figure 5, treatment of SH-SY5Y cells with microglial conditioned medium had no significant effect on the expression of either APP or BACE-1. In contrast, conditioned medium from iron-fed microglia but not control microglia had a significant effect reducing the levels of ADAM10 protein by SH-SY5Y cells.

We investigated the consequences of altered ADAM10 expression through a variety of assays. First we used a dual luciferase assay of APP cleavage to assess if there is any change in the rate of formation of the AICD fragment of APP. In this system, a GAL4 DNA binding tag is attached to the C-terminus of the APP protein. The assay is dependent on the cleavage of this tagged form of APP and the release of the tag,

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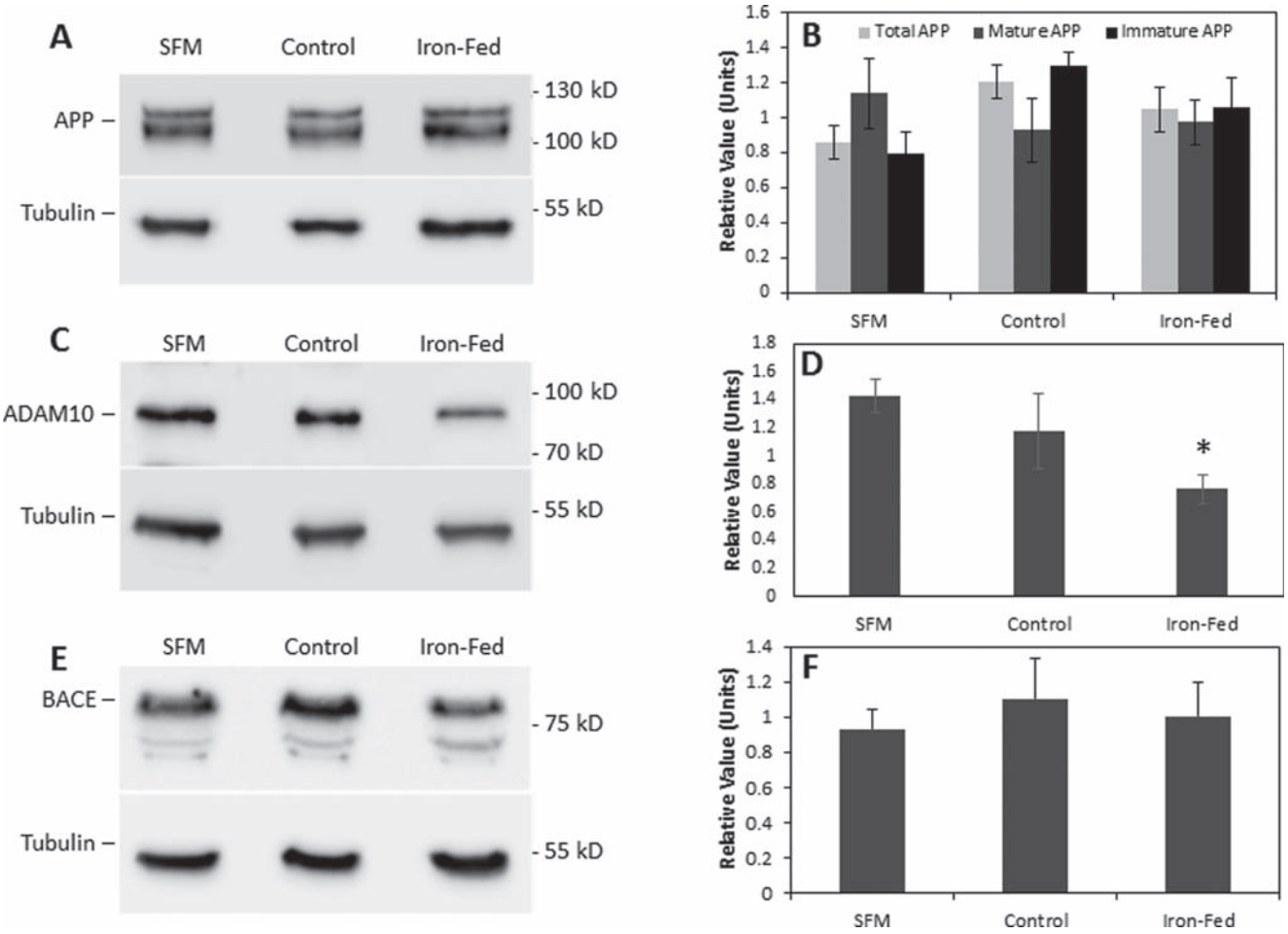

Figure 5. Effects of microglial conditioned medium on protein expression. Conditioned medium was prepared from control and iron-fed microglia. The conditioned medium was applied to SH-SY5Y cells for 24 h. Extracts were prepared from the SH-SY5Y cells along with cells that had been treated only with serum free medium (SFM). The extracts were applied to a PAGE gel, and the gel was blotted to a membrane. APP, ADAM10, BACE-1, and tubulin (loading control) were detected on the resultant membrane with specific antibodies. Following detection, the intensity of the bands was assessed using densitometry. (A, B) Analysis of APP expression in SH-SY5Ys showed there was no significant ($p > 0.05$) effect of either control or iron-fed conditioned medium. The same result was seen whether the individual bands were quantified together or separately. (C, D) In contrast, treatment of SH-SY5Y cells with conditioned medium from iron-fed microglia (but not control) reduced the protein expression of ADAM10 significantly ($p < 0.05$) when compared to SFM. (E, F) There was no significant effect of either microglial conditioned medium on the expression of BACE-1 in SH-SY5Y cells. Shown are the mean and SE for four experiments each. * indicates a significant difference when compared to SFM.

which is then able to induce luciferase expression by binding to the luciferase reporter. Our previous work has demonstrated that in SH-SY5Y cells, the predominant cleavage of this tagged APP is sequential via β-secretase and then γ-secretase to release the tagged AICD fragment.[40] SH-SY5Y cells were transiently transfected with the APP expression plasmid, the luciferase reporter plasmid, and a third *Renilla* luciferase construct that controls for transfection efficiency and differences in cell number. The transfected cells were then treated with conditioned medium from control and iron-fed microglia for 24 h. Luciferase activity was then measured in extracts from the cells and compared to transfected controls that were treated only with serum free medium. The result showed that medium from both control and iron-fed microglia had a significant effect on the luciferase levels detected (Figure 6A). This suggests that AICD fragment formation is greatly reduced by microglia conditioned medium. However, there was no significant difference between control and iron fed conditioned medium. This implies that the change in ADAM10 expression induced by iron-fed microglia had no effect on rates of APP cleavage.

We then measured the activity of the ADAM10 promoter using a reporter construct containing the promoter for human ADAM10. The SH-SY5Y cells were similarly transiently transfected with this construct and the *Renilla* control. The transfected cells were then treated with conditioned medium from control and iron-fed microglia for 24 h. The result showed that neither medium from control nor iron-fed had any significant effect on the luciferase levels detected (Figure 6B). This implies that the change in ADAM10 protein expression that we observed was not a consequence of altered transcription of ADAM10 mRNA.

Following from this, we wished to confirm whether altered ADAM10 expression resulted in altered ADAM10 activity. We therefore used a FRET-based assay to assess ADAM10 activity in extracts of SH-SY5Y cells treated with conditioned medium from control and iron-fed microglia. The assay measured the cleavage of a tagged peptide substrate. After 24 h of treatment

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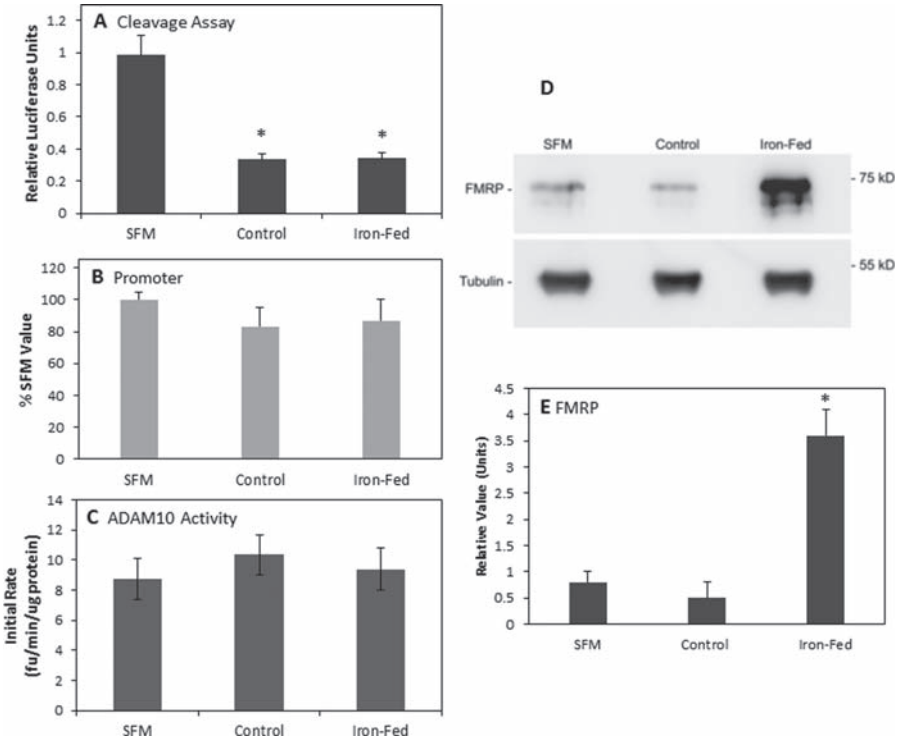

Figure 6. Investigation of microglia induced changes in ADAM10. (A) The level of APP cleavage in SH-SY5Y cells was measured using a dual luciferase reporter assay. The assay is based upon binding of a GAL-4 DNA binding domain to the DNA sequence in the luciferase reporter construct. Binding is directly proportional to the release of the ACID domain from APP to which the GAL-4 domain is fused. SH-SY5Y cells transfected with the reporter constructs were treated for 24 h with conditioned medium from control and iron-fed microglia or with serum free medium (SFM). Activity measured from the firefly luciferase reporter construct reporting AICD release was divided by *Renilla* luciferase control activity (pTK) to give relative luciferase activity for each treatment. Conditioned medium from both control and iron-fed microglia caused a significant reduction in the luciferase activity measured ($p < 0.05$) compared to SFM. However, there was no significant difference when comparing the effect of control and iron-fed microglia conditioned medium to each other. (B) The level of ADAM10 promoter activity was assessed in SH-SY5Y cells. The cells were transiently transfected with the luciferase reporter construct carrying the ADAM10 promoter and the control reporter (pTK). The cells were treated with conditioned medium from control and iron-fed microglia for 24 h. The level of luciferase activity was then assessed and compared to that measured in the SFM control. Neither control nor iron-fed microglial conditioned medium had a significant ($p > 0.05$) effect on luciferase activity. (C) The level of ADAM10 activity in SH-SY5Y cells was assessed with a commercial kit. The kit measured fluorescent activity following cleavage of an ADAM10 substrate by ADAM10 present in extracts from the cells. Extracts were prepared from SH-SY5Y cells treated for 24 h with conditioned medium from control or iron-fed microglia or SFM. Fluorescence was measured at 520 nm in a plate reader following application of extract to the assay. The values were adjusted to the protein concentration of the extracts. Treatment of SH-SY5Y cells with microglia conditioned medium had no significant effect ($p > 0.05$) on measured ADAM10 activity. (D, E) FMRP is a translation inhibitor known to influence protein expression of ADAM10. We measured FMRP expression in protein extracts from SH-SY5Y cells by western blot. SH-SY5Y cells were treated with conditioned medium from control and iron-fed microglia or SFM. After 24 h, protein extracts were prepared from the SH-SY5Y cells and applied to the western blot procedure. FMRP and tubulin (loading control) were detected with specific antibodies, and the band intensity after chemiluminescence detection was assessed with densitometry. Conditioned medium from iron-fed microglia but not control microglia had significant effect on FMRP detected in SH-SY5Y cells ($p < 0.05$), greatly increasing the level detected. Shown are the mean and SE for four experiments in all cases. * indicates a significant difference when compared to SFM.

with conditioned medium, extracts from the treated SH-SY5Y cells were applied to the assay, and the level of cleavage was measured. The results showed that there was no significant difference in ADAM10 activity in SH-SY5Y cells treated with conditioned medium from microglia (Figure 6C). The implication of this is that, despite the change in ADAM10 protein expression, there is no consequence of this in terms of measurable activity.

Lastly, as we have observed reduced protein expression of ADAM10, we looked for an alternative explanation for the change. It is known that ADAM10 translation can be down-regulated through the activity of the translation suppressor FMRP (fragile-X mental retardation protein).[41] We assessed the expression of FMRP in SH-SY5Y cells by western blot. SH-SY5Y cells were treated with conditioned medium from control and iron-fed microglia for 24 h. Protein extracts were then prepared, and the western blot procedure was carried out. The levels of FMRP were assessed using a specific antibody. The results showed a significant increase in FMRP in SH-SY5Y cells treated with conditioned medium from iron-fed microglia but not from control microglia (Figure 6D,E). What this points to is that conditioned medium from iron-fed microglia increases the expression of the translation inhibitor FMRP known to regulate the translation of ADAM10.

The cleavage of APP causes release of β-amyloid from neuronal cells such as SH-SY5Y cells. The released β-amyloid can be detected in cell culture medium. We used a very sensitive MSD immunoassay to measure β-amyloid released into the serum free cell culture medium by SH-SY5Y cells. Treatment of SH-SY5Y cells with conditioned medium from

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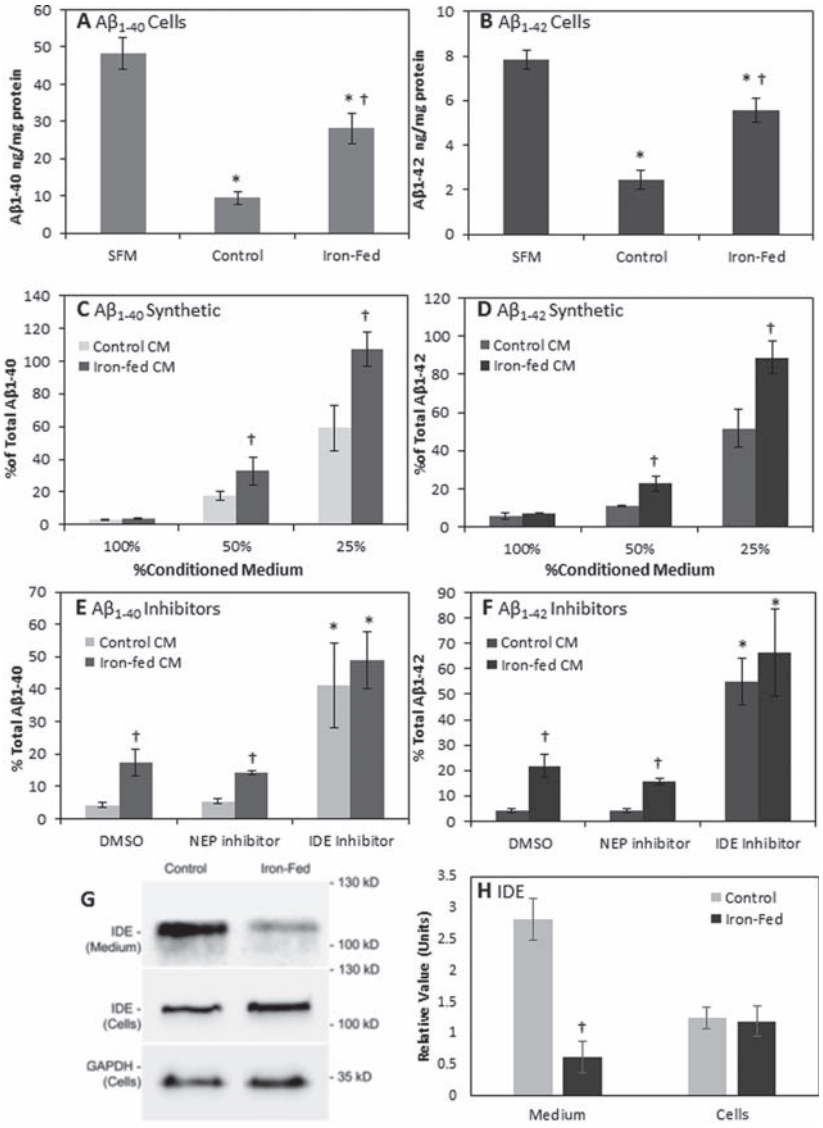

Figure 7. Microglia degradation of β-amyloid. We used an MSD Sector Imager to measure the levels of β-amyloid in culture medium. The multiplex MSD assay measured both Aβ_{1-40} and Aβ_{1-42} simultaneously in the same experiments. (A, B) SH-SY5Y cells were treated with conditioned medium from control and iron-fed microglia. The levels of β-amyloid released after 24 h were assessed and compared to that of SH-SY5Y cells grown in serum free medium alone (SFM). The levels of both Aβ_{1-40} and Aβ_{1-42} were significantly ($p < 0.05$) reduced after either treatment. However, the levels of both peptides were significantly higher when SH-SY5Y cells were treated with medium from iron-fed microglia than when they were treated with medium from control microglia. (C, D) Synthetic Aβ_{1-40} and Aβ_{1-42} were treated with conditioned medium from control and iron-fed microglia or SFM for 24 h. The conditioned medium was diluted with SFM. After this time, the levels of β-amyloid remaining were assessed with the MSD assay. In the SFM controls, 904 ± 141 ng/mL Aβ_{1-40} and 157 ± 27 ng/mL Aβ_{1-42} were detected. The amount remaining in the conditioned medium treated wells is shown as a percentage of these values. At both 50% and 25% conditioned medium, there was significantly more Aβ_{1-40} and Aβ_{1-42} remaining under iron-fed conditions than control ($p < 0.05$) implying that conditioned medium from iron-fed microglia degraded both forms of β-amyloid less than control microglial conditioned medium. (E, F) The experiment with synthetic Aβ_{1-40} and Aβ_{1-42} was repeated but with the addition of enzyme inhibitors. Aβ_{1-40} and Aβ_{1-42} were treated with conditioned medium from control and iron-fed microglia with the addition of DMSO vehicle, IDE inhibitor, or neprylisin (NEP) inhibitor. After 24 h, the levels of Aβ_{1-40} and Aβ_{1-42} remaining were assessed with the MSD assay. In the SFM controls, 3715 ± 385 ng/mL Aβ_{1-40} and 246 ± 42 ng/mL Aβ_{1-42} were detected. Compared to the DMSO control, the NEP inhibitor had no significant effect on the levels detected for either control or iron-fed conditioned, while the IDE inhibitor significantly reduced degradation of both Aβ_{1-40} and Aβ_{1-42} ($p < 0.05$). (G, H) The levels of IDE were measured in both control and iron-fed microglia and also in the conditioned medium generated from these cells. The relative levels of IDE were measured using western blotting and detection with a specific antibody. GAPDH was measured as a control for loading of the cell lysates. The conditioned media from the microglia were concentrated 10-fold with a 30 kDa centrifugal filter (Sartorius) to increase chances of detection. After western blotting the detected bands were densitometrically quantified. The levels of IDE in the cells were not significantly different between control and iron-fed microglia while that released into the medium was significantly ($p < 0.05$) reduced for iron-fed microglia. For all sections shown are the mean and SE for at least four independent experiments. * indicates a significant different between treatment and control. † indicates a significant difference between treatments with conditioned medium from control and iron-fed microglia.

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control microglia for 24 h resulted in a large and significant reduction in β-amyloid (both $A\beta_{1-40}$ and $A\beta_{1-42}$) that could be detected in the medium (Figure 7A). In comparison, SH-SY5Y cells treated with conditioned medium from iron-fed microglia had a significantly reduced effect on the levels of β-amyloid (Figure 7A,B). The implication of this result is that control microglia are more able to reduce the levels of β-amyloid released by SH-SY5Y cells than iron-fed microglia. It should be noted that treatment of SH-SY5Y cells with just iron had no significant effect on β-amyloid levels (Supporting Figure 1).

The decreased levels of β-amyloid detected in medium of SH-SY5Y cells following treatment with microglial conditioned medium can either be a result of decreased release of β-amyloid or increased breakdown of the peptide. While we have shown that conditioned medium from microglia reduced APP cleavage, there was no significant difference between control and iron-fed microglia. Therefore, the difference we noted in β-amyloid present in the medium is more likely to be a result of altered breakdown. We therefore used synthetic β-amyloid to measure its breakdown by microglia conditioned medium. Equal amounts of β-amyloid was placed in wells and treated with either serum free medium, control microglia conditioned medium, or iron-fed microglia conditioned medium for 24 h. Following the assay, the levels of β-amyloid measured were compared as a percentage to the levels detected in the serum free medium control as a percentage. Three different concentrations of the conditioned medium were tested. β-amyloid (both $A\beta_{1-40}$ and $A\beta_{1-42}$) was degraded by the conditioned medium in direct relation to the amount of conditioned medium added (Figure 7C,D). At 25% and 50% concentrations, conditioned medium from iron-fed microglia had a significantly weaker effect. The implication is that microglia release factors in their conditioned medium that degrade β-amyloid and iron-fed microglia release significantly less of these.

Microglia release a number of factors known to degrade β-amyloid. Among these are insulin-degrading enzyme (IDE)[42] and neprilysin.[43] We tested specific inhibitors of these enzymes to determine what part of the reduction in β-amyloid caused by microglial conditioned medium was due to the presence of these enzymes. We repeated the MSD assay for testing the degradation of β-amyloid by conditioned medium but added either 30 μM ML345 (IDE inhibitor)[44] or 10 μM thiorphan (neprilysin inhibitor)[45] in parallel. The results show that the IDE inhibitor but not the neprilysin inhibitor significantly reduced the degradation of β-amyloid (both $A\beta_{1-40}$ and $A\beta_{1-42}$) by microglia conditioned medium (Figure 7E,F). This implies that IDE is the likely candidate protein released from microglia that degrades β-amyloid in our system.

We verified that IDE was released by control and iron-fed microglia by collecting conditioned medium from microglia, concentrating it, and testing for the presence of IDE by western blot. IDE was detected at equivalent expression levels in control and iron-fed microglia and was also present in the medium collected from the cells (Figure 7G,H). The levels of IDE in iron-fed microglial conditioned medium were significantly lower than for control. This supports the suggestion that iron-fed microglia release significantly less IDE than control microglia.

Mechanism of Reduced IDE Release by Iron-Fed Microglia. We observed no change in IDE expression in iron-fed microglia. Therefore, another factor must be altering the levels released. It has been reported that IDE released from microglia is influenced by changes in autophagy.[46] Therefore, we assessed autophagic flux in our model microglia. The expression levels of the protein LC3-II are a reliable indicator of the level of autophagosomes in cells.[47] Inhibition of autophagy with bafilomycin in conjunction would then give an assessment of the turnover of LC3-II.[48] Therefore, an increase in LC3-II levels in microglia that remains unaltered by bafilomycin treatment would be indicative of reduced autophagy. Control and iron-fed microglia were grown in serum free medium and treated either with 13 nM bafilomycin or the equivalent volume of methanol vehicle (20 μL). After 16 h, protein extracts were prepared from the cells, and the levels of LC3-I and LC3-II were determined by western blot (Figure 8). Iron-fed microglia grown in serum free medium showed

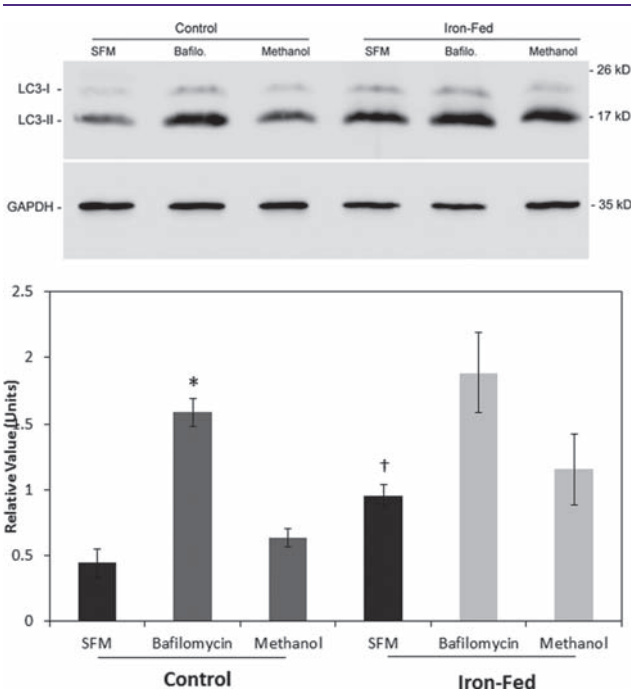

Figure 8. Autophagy in iron-fed microglia. The levels of LC3-II were determined in human microglia by western blot. Control and iron-fed microglia were grown in serum free medium (SFM) and treated either with 13 nM bafilomycin (Bafilo) or the equivalent volume of methanol (20 μL, 0.67% of total volume). After 16 h, protein extracts were prepared from the cells and applied to a 14% PAGE gel. After transfer to a membrane, the presence of LC3-II and GAPDH (loading control) was detected with specific antibodies. After chemiluminescent detection to visualize the bands (upper panel), the intensity of the bands was assessed with densitometry. Iron-fed cells grown in SFM showed significantly higher levels of LC3-II than control microglia ($p < 0.05$). Treatment with bafilomycin significantly increased the levels of LC3-II for control microglia but not for iron-fed microglia. Shown are the mean and SE for seven experiments. * indicates a significant difference between treatment and methanol control. † indicated a significant difference between control and iron-fed microglia.

significantly higher LC3-II levels than control microglia. Bafilomycin significantly increased the levels of LC3-II detected in control microglia when compared to the methanol control, but there was no such change for iron-fed microglia with the same treatment. The results suggest that autophagy in iron-fed microglia is inhibited in comparison to control microglia.

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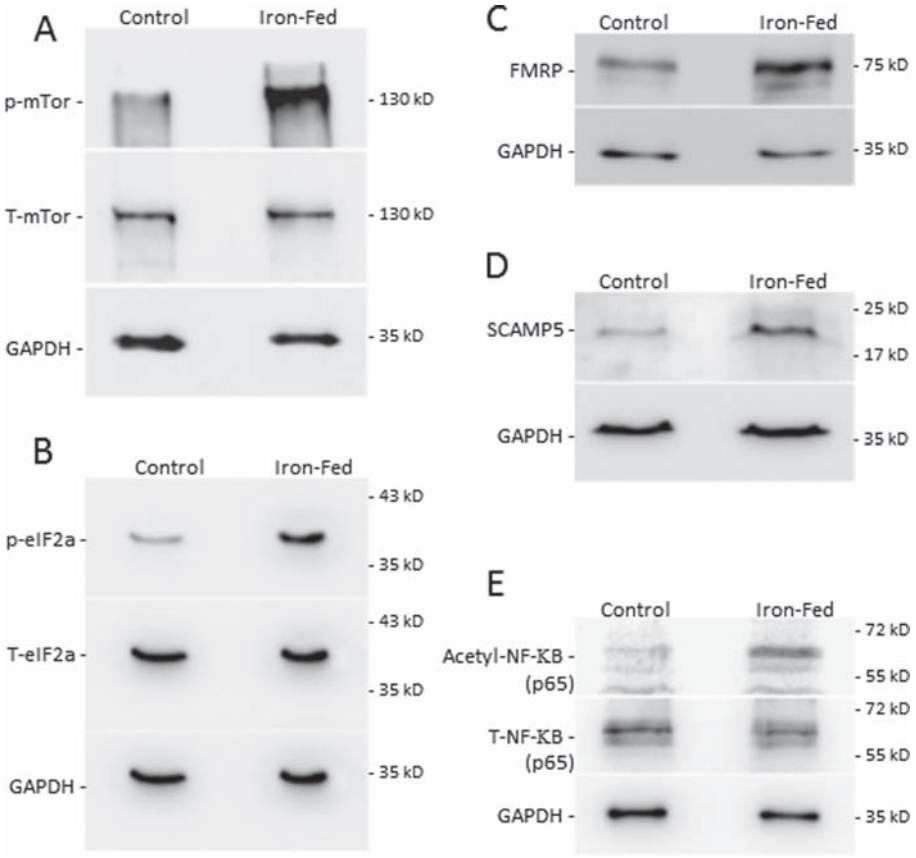

Figure 9. Expression of proteins related to autophagy and ER stress. Protein extracts were prepared from control and iron-fed microglia. western blotting and immunodetection were used to assess the level of expression of a range of proteins. These proteins were (A) phosphorylated-mTOR (p-mTOR) and total mTOR (T-mTOR), (B) phosphorylated-EIF2a (p-EIF2a) and total EIF2a (T-EIF2a), (C) FMRP, (D) SCAMP5, and (E) acetyl-NF-κB and total NF-κB (T-NF-κB). In each case, GAPDH was also analyzed to ensure equal loading of the samples. Quantitation appears in Table 2.

Changes in autophagy are often associated with changes in endoplasmic reticulum (ER) stress. Mechanistically, this would provide a potential start point for the changes observed as ER stress can be induced by iron overload.[49] We therefore examined the expression of a range of proteins that either link or are associated with ER stress and autophagy. These include mTOR (mammalian target of rapamycin) associated with down-regulation of autophagy when phosphorylated,[50] EIF2a (eukaryotic translation initiation factor 2a) associated with increased ER stress,[51] SCAMP5 (secretory carrier membrane protein 5), which links increased ER stress to decreased autophagy,[52] FMRP, which decreases the expression of proteins such as SIRT1 as a result of ER stress,[53] and acetylated NF-κB, which is associated with alteration in released cytokines as a result of ER stress.[54] Extracts were prepared from control and iron-fed microglia and applied to the western blot procedure. Specific antibodies were used to detect bands associated with the proteins of interest (Figure 9), and the bands were quantified by densitometry (Table 2). There was a significant increase in the level of phosphorylated mTOR, but the levels of total protein remained the same. This change fits with the suggestion that autophagy is down regulated in iron-fed microglia. Similarly, levels of phosphorylated EIF2a were increased with no change in the total protein. This fits with the notion that ER stress is increased in iron-fed microglia. Both FMRP and SCAMP5 were increased further supporting these suggestions. Lastly, acetylated NF-κB (p65) was increased with no change in total NF-κB levels. This change is also associated with decreased autophagy through decreased SIRT-1 activity.[55]

Table 2. Altered Expression of Protein in Iron-Fed Microglia[a]

protein	control	iron-fed
total mTOR	1.6 ± 0.2	1.8 ± 0.2
ratio p-mTOR/T-mTOR	1.0 ± 0.1	4.2 ± 0.7[b]
total eIF2a	1.1 ± 0.2	1.0 ± 0.1
ratio p-eIF2a/T-eIF2a	1.0 ± 0.3	2.3 ± 0.3[b]
FMRP	1.0 ± 0.2	2.3 ± 0.3[b]
SCAMP-5	1.0 ± 0.2	2.4 ± 0.4[b]
total NF-κB	1.1 ± 0.2	1.0 ± 0.2
ratio acetyl-NF-κB/T-NF-κB	1.0 ± 0.3	2.6 ± 0.7[b]

[a]Densitometric analysis of the bands on western blots shown in Figure 10. Total levels of mTOR, eIF2a, and NF-κB were assessed as well as the ratio of the modified (p or acetyl) form to the total form (T). Shown are mean and SE for 4−8 experiments each. [b]Significant difference between control and iron-fed ($p < 0.05$).

As we have evidence that both autophagy and ER stress are altered in iron-fed microglia, we wished to determine if inhibiting autophagy and inducing ER stress would cause a change in IDE release from control microglia. We therefore used bafilomycin to inhibit autophagy and both brefeldin A and tunicamycin to induce ER stress. Conditioned medium

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was collected from control microglia that had been treated overnight with these compounds, and extracts were prepared from the treated microglia to assess cellular levels of IDE. The conditioned medium was concentrated, and the IDE levels were assessed by western blot (Figure 10). All three compounds significantly reduced the levels of IDE that could be detected in conditioned medium but had no effect on the cellular levels of IDE. These results support the notion that ER stress and consequential reduced autophagy cause reduced levels of IDE release by microglia.

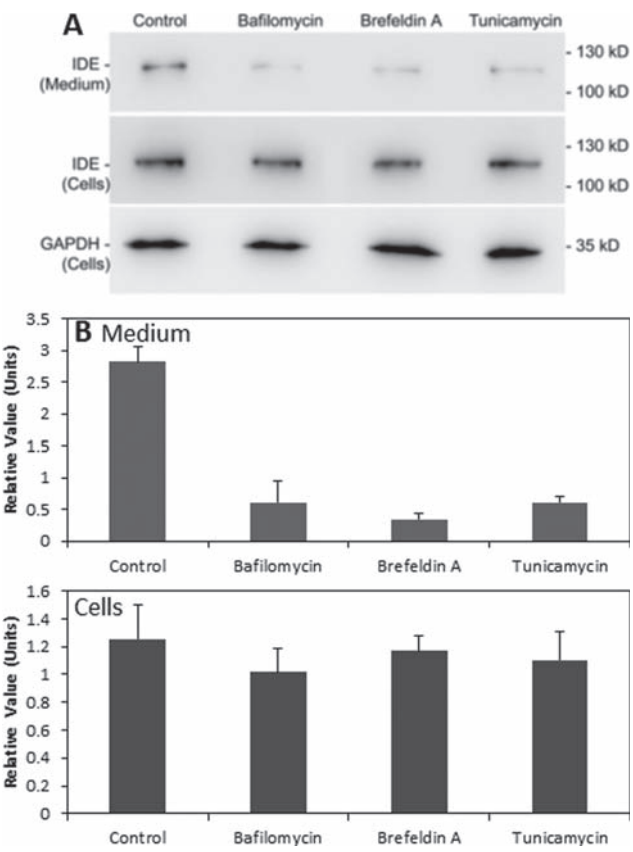

Figure 10. Autophagy, ER stress, and IDE release. The levels of IDE were measured in cell extracts from control microglia with and without treatment with either with 13 nM bafilomycin, 0.1 μg/mL brefeldin A, or 1.0 μg/mL tunicamycin. The treatments were for 16 h, and after that time conditioned medium generated from these cells was collected, and protein extracts were made from the cells. The relative levels of IDE were measured using western blot and detection with a specific antibody. GAPDH was measured as a control for loading of the cell lysates. The conditioned media from the microglia were concentrated 10-fold to increase chances of detection. After western blot, the detected bands were densitometrically quantified. None of the treatments increased IDE levels in the microglia, but all treatments caused a significant decrease ($p < 0.05$) in IDE detected in the medium when compared to the control. Shown are the mean and SE from seven independent experiments.

Lastly, to verify that the effect of autophagy inhibition and ER stress diminishes the ability of microglia to degrade β-amyloid, the conditioned medium from the microglia treated with bafilomycin, brefeldin A, or tunicamycin was applied to the MSD assay using synthetic β-amyloid. β-Amyloid was treated with the conditioned medium from the microglia for 24 h, and the levels of β-amyloid remaining were assessed. Treatment of the microglia with all three compounds significantly decreased the degradation of β-amyloid (both $A\beta_{1-40}$ and $A\beta_{1-42}$), indicating that reduced IDE release resulted in decreased β-amyloid degradation (Figure 11).

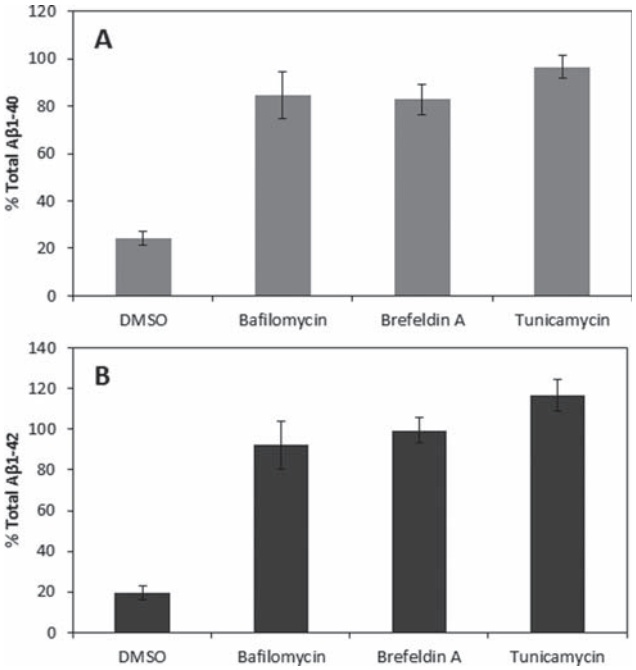

Figure 11. Autophagy, ER stress, and β-amyloid. Synthetic $A\beta_{1-40}$ and $A\beta_{1-42}$ were treated with conditioned medium from control microglia treated with 13 nM bafilomycin, 100 ng/mL brefeldin A, 1.0 μg/mL tunicamycin, or SFM alone for 16 h. After this time, the levels of β-amyloid remaining were assessed with the MSD assay. In the SFM controls, 3326 ± 240 ng/mL $A\beta_{1-40}$ and 231 ± 10 ng/mL $A\beta_{1-42}$ were detected. All three treatments significantly increased ($p < 0.05$) the level of $A\beta_{1-40}$ and $A\beta_{1-42}$ detected when compared to control microglia conditioned medium. Shown are the mean and SE for four experiments.

DISCUSSION

Nonfamilial AD is the most common neurodegenerative disease, and incidence rapidly increases with age, making the aging process the major risk factor.[56] Therefore, understanding how brain aging impacts the incidence of AD is a major necessity for its possible treatment. While the exact cause of AD remains unresolved, the deposition of aggregates of β-amyloid is at the least a significant marker for its progression and at the most the causative agent.[57] The true role of β-amyloid in the pathogenesis probably lies somewhere between these extremes. While the formation of β-amyloid is a normal cellular consequence of the metabolic breakdown of APP, increasing its levels either through alterations in the metabolic fate of APP or increasing its extracellular survival through reduced breakdown directly corresponds to increased possibility of extracellular aggregates forming.[58−60]

There has been increasing discussion of the potential role of microglial aging and dysfunction in AD.[20,28,61−63] However, the nature of patient tissues and even animal models make assessment of molecular interactions difficult, necessitating cell models where real time interactions can be monitored and changes assessed. Our model of dystrophic/senescent microglia therefore provides an opportunity for which there is

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currently little alternative. Given the issues with rodent models to reproduce an inherently human disease,[64] we deliberately chose human cell lines for our approach. While different microglia "states" such as activated and primed[65] or phenotypes such as ramified and phagocytic can be modeled, there are no such models for senescent/dystrophic microglia. The major criterion for determining the characteristics of dystrophic microglia is based on morphological changes in tissue, which inherently poorly translates to *in vitro* models.[18,35] Similarly, there is no universal marker that can discriminate a dystrophic microglia from any other.[18] Worse still is that large scale proteomics/transcriptomics based studies have failed to identify reproducible differences between aged microglia and controls, with each study listing almost incomparable data sets.[66−71] The one exception to this is ferritin expression. Microglia express more ferritin than most cells of the brain, but this is further elevated in dystrophic microglia and implicates that iron storage is also increased.[22] A more objective way to assess senescence in microglia is to use the same criteria used for assessing general cellular senescence, that is, the characterization of their secretory phenotype.[72] The induction of the senescence associated secretory phenotype (SASP) is likely to become the standard approach to identify senescent cells in the absence of a specific marker.[73]

We induced a senescent/dystrophic phenotype in microglia by iron overload. This resulted in a change in expression of ferritin and iron storage. While the high levels of iron used were artificial and unlikely to be encountered *in vivo*, they were employed as a means to an end and played no further role once we had induced the phenotype, as the high iron environment was removed from the culture system when analyzing changes and producing conditioned medium. However, the levels of stored iron and the increase in ferritin levels that we measured are in line with previous suggested increases in both iron concentrations and ferritin levels in specific brain regions that have previously been measured.[74] Of all the cell types in the brain, microglia show the largest increase in iron storage with age and are therefore likely to have much higher levels than the average for any specific brain region.[25]

We verified the senescent/dystrophic phenotype by measuring molecules released by the microglia (cytokines and glutamate),[36,75] as well as changes in protein expression associated with an aged phenotype (KV1.3, SIRT-1).[37,39] Interestingly age associated changes in both KV1.3 and SIRT-1 have been shown to influence increased cytokine release.[38,39] Other changes observed later in the study also support the SASP state of the iron-fed microglia. These include changes in autophagy and ER stress.[76,77]

The multitude of changes we have measured in our model microglia converge on a general phenotype that would be expected in a senescent cell. Along with altered release of molecules such as cytokines, these changes included reduced ability to deal with stress and a failure in processes aimed at clearing away damaged proteins such as autophagy.

The relation of microglia to AD has long been considered.[78−81] Similar to the study of many neurodegenerative diseases, the study of microglia in AD has principally concerned the physical relation of microglia in patient brains and transgenic mice to pathological hallmarks such as β-amyloid deposition[82] or neuronal loss.[83] The potential role of microglia has been suggested to be direct (causing neuronal loss through activation in response to β-amyloid),[84] indirect (changing parameters such as plague load),[85] or bystander (attracted to plaques without altering the pathology). There have been some suggestions that β-amyloid is not necessary to the mechanism of neuronal loss and that eliminating microglia in AD transgenic mice prevents neuronal loss without change to β-amyloid plaque levels.[86] However, the most convincing evidence suggests that microglia influence clearance of β-amyloid due to the production of enzymes able to degrade it or by phagocytic uptake.[32] Thus, regulation of β-amyloid clearance is the most likely physiological role of microglia in AD.

Our findings emphasize the importance of microglial proteases to the removal of β-amyloid and how disruption of the release of enzymes like IDE could compromise this role. The human microglia cell line we used released IDE, which in our system almost completely removed β-amyloid released by SH-SY5Y cells over 24 h. IDE is released by primary microglia but not all microglia cell lines (e.g., BV2), indicating that caution must be used in the choice in cell lines to model age-related microglia changes.[87,88] The reduced release in IDE we observed for iron-fed microglia was a result of reduced autophagy, which has been previously observed,[46,89] but is a mechanism that has been poorly studied. It has been noted that changes in this pathway involve changes in the level of phosphorylated mTOR,[89] which we also observed. Outside of the possible association of reduced IDE release with the dystrophic phenotype, we observed that both reduced autophagy and increased ER stress had the same effect. The implication is that any process that alters microglia in a similar way could also influence the levels of β-amyloid in the brain. However, reduced autophagy and increased ER stress are characteristics of senescence-associated secretory phenotype.[76,77] It should also be noted that the change in IDE release was not due to a general failure in IDE expression in the cells as we did not observe any change in cellular expression.

There has been discussion of how important reduction in β-amyloid degrading enzymes is to AD. There are some reports that suggest that such a reduction would only contribute to late stage changes.[90] Of course, studies of patients do not take into account preclinical changes that may play a role in disease onset. The general consensus is that these enzymes are extremely important and regardless of causality, regulating their activity may have therapeutic value.[91] An additional consideration is the relative contributions of the different enzymes to β-amyloid degradation in the brain. Our study has focused on IDE and not neprilysin because we found no contribution from the latter in our system, but this may not reflect the roles of these enzymes in AD. There has been suggestion that neprilysin is the main enzyme affected in AD,[92] and others suggest it might be a different enzyme altogether, such as angiotensin-converting enzyme.[93] However, IDE is clearly important when considering β-amyloid degradation in the brain. First, IDE is reduced with age in regions associated with AD.[94] Second, overexpression of IDE causes a significant reduction in plaque load.[95] Third given these enzymes are both released by microglia and as the principal change may be in the SASP of microglia it may be a moot point exactly which enzyme is most involved.

A final additional factor is that enzymes generated by neurons themselves and released extracellularly might also contribute to APP degradation. Such enzymes include endothelin-converting enzyme and membrane type metallomatrix proteins (MMPs).[96,97] These enzymes may also be released by SH-SY5Ys. Factors from microglia may also

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influence levels of these proteins, which could have also affected levels of β-amyloid in our system.

We also examined other factors that could alter the generation of β-amyloid by SH-SY5Y cells. In particular, we noted that conditioned medium from microglia significantly reduced the formation of AICD, a C-terminal cleavage product of APP, but this effect was replicated when the conditioned medium was generated from iron-fed microglia. While a change in the processing of APP could result in reduced release of β-amyloid by SH-SY5Y cells and consequently the level of β-amyloid measured in the MSD assay, it has no bearing on the assays with synthetic β-amyloid. Therefore, these results support the potential role of microglia in regulating β-amyloid levels released from cells. Destruction of microglia in mice has been shown to increase plaque size.[98]

We also noted a significant change in the levels of ADAM10 expression in SH-SY5Y cells treated with conditioned medium from iron-fed microglia, but as there was no observed change in the activity of ADAM10, the consequences of this altered expression are likely to be insignificant to the processing of APP and the subsequent levels of β-amyloid. These findings do not rule out other potential changes to protein expression and metabolism in SH-SY5Y cells, but these may or may not have any bearing on APP metabolism specifically. While the study on microglia−neuron interactions is hardly new, there has been little study of the impact of microglia on APP processing in neurons. This is surprising given there is significant evidence that APP processing is influenced by cytokines released by microglia.[99−101] However, we are confident that a system of study such as ours will increase consideration of microglia in the study of APP.

In summary, we have established a unique model of senescent/dystrophic microglia and used this to examine the role of dystrophic microglia in the turnover of β-amyloid, a major player in AD. A comprehensive overview of our findings is illustrated in Figure 12. Microglia induced to take up iron show changes in molecular expression suggestive of changes in ER stress and autophagy. The consequence of these changes is the adoption of a senescence associated secretory phenotype and changes in the release of a variety of proteins including cytokines and enzymes like IDE. Decreased release of IDE results in decreased breakdown of β-amyloid released by neuronal cells. These findings provide a potential mechanistic insight into how microglial aging can contribute to the accumulation of β-amyloid and advance the pathology of AD.

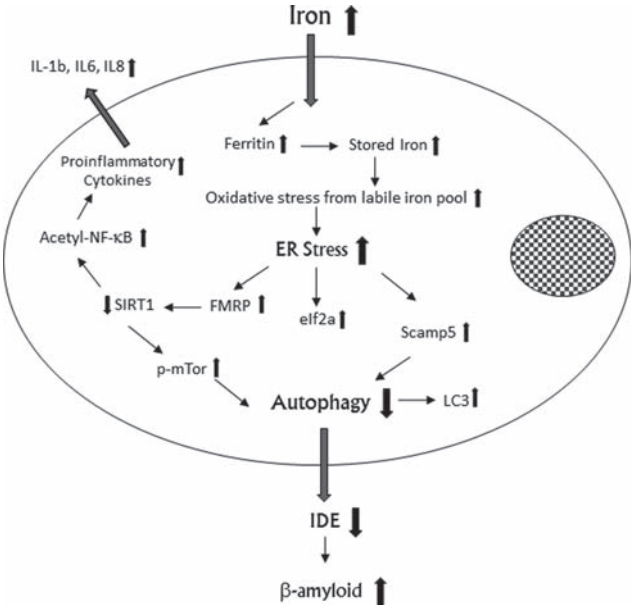

Figure 12. Model of dystrophic microglia induced increase in β-amyloid. A summary figure illustrating the changes we have observed in this study. Excess uptake of iron by microglia induces a dystrophic-like phenotype illustrated by changes in ferritin expression and iron storage. Increased cellular iron also increases the labile iron pool with the consequence of increased oxidative events, which are known to induce ER stress. The consequence of induced ER stress is changes in markers such as SCAMP5 and EIF2a and altering protein translation through FMRP. One consequence of induced action of FMRP is the decreased expression of the deacetylase SIRT-1 resulting in higher levels of acetylated NF-κB. This change is known to result in increased release of pro-inflammatory cytokines indicating a switch in the secretory phenotype to one associated with cell senescence. Decreased SIRT1 activity is also associated with increased phosphorylation of mTOR, resulting in reduced autophagy. Decreased autophagy causes an increase in the levels of LC3-II by reduction in its turnover. It also results in a decrease in the secretion of proteins that include IDE. Reduced IDE then results in increased extracellular levels of β-amyloid.

METHODS

Unless otherwise stated reagents were purchased from Sigma-Aldrich.

Cell Culture. The microglia cell line used in this study was the SV40 immortalized human microglia cell line (ABM). Cells were cultured in DMEM with 4.5 g/L glucose (LONZA) supplemented with 10% FBS (Labtec) and 1% pen/strep. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. The neuronal cell line used in experiments was the SH-SY5Y human neuroblastoma. This cell line was grown under the same conditions as the microglial cell line. Images of microglia were produced using either a Nikon Diaphot-TMD inverted microscope (live cells) or a Nikon Eclipse E800 microscope (stained cells). Perl's staining was performed using a kit from Atom Scientific. Control and iron-fed microglial cells were plated onto coverslips and left overnight. They were washed in PBS and fixed in ice-cold methanol for 5 min at −20 °C. The cells were stained according to the manufacturer's instructions and mounted onto slides using glycerol jelly as mounting medium.

Microglia were cultured in high iron conditions to induce a senescent phenotype. The iron stock solution used was 25 mM ferric ammonium citrate (Acros Organics) prepared in deionized water and filtered through a 0.22 μm syringe filter (Millipore) Microglial cell lines were grown in medium containing in 500 μM ferric ammonium citrate for a minimum of 2 weeks. Cell lines were maintained under these conditions until used for experiments. Conditioned medium was produced from the microglia by washing them with serum free medium (DMEM) to remove excess iron and then growing them for 48 h in DMEM supplemented with B27 without antioxidants (Gibco) plus 1% penicillin and streptomycin. The conditioned medium was collected from both control and iron-fed microglia, centrifuged to remove debris, and filtered through a 0.45 μm filter before use in further experiments.

Drug treatments were for 16 or 24 h. Brefeldin A (10 mM), bafilomycin (2 μM), and tunicamycin (1.2 mM) were prepared as stock solutions in methanol. Concentrated stocks of the IDE inhibitor ML345 (10.4 mM) and the neprilysin inhibitor thiorphan (1 mM) were prepared in DMSO. Synthetic β-amyloid was from Meso Scale Discovery.

Western Blot. Cells were lysed in PBS with 0.5% Igepal CA-630 and "complete" protease inhibitor cocktail (Roche), sonicated 5 s on ice, incubated on ice for 20 min, and centrifuged 10 000*g* for 5 min to remove insoluble membranes. Protein concentration was determined with a Bradford protein assay (Bio-Rad), according to the

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manufacturer's instructions. Protein concentrations were normalized, and samples were boiled for 5 min with 1× Laemmli SDS-PAGE buffer. Samples were loaded onto either a 10% or a 14% (depending on molecular weight of the protein) acrylamide SDS-PAGE gel, with a buffer of Tris (250 mM), glycine (1.92 M), and SDS (0.1% w/v), and run at 250 V and 35 mA/gel for 45−60 min. Separated proteins were transferred to a PVDF membrane (Millipore) using a semidry transfer apparatus, run at 25 V and 100 mA/gel for 1.5 h. Membranes were blocked in 5% w/v nonfat milk powder in Tris buffered saline with 0.1% Tween 20 (TBS-T) for 1 h, incubated with primary antibody for 1−2 h or overnight, and washed 3 × 15 min in TBS-T. Membranes were blocked again for 10 min and incubated with horseradish peroxidase-conjugated secondary antibody (DAKO) for 1 h. Additional 3 × 15 min washes were performed, and the membranes were developed with Luminata Crescendo or Luminata Forte ECL substrate (Thermo Scientific) and imaged with a Fusion SL CCD imaging system (Vilber Lourmat). Antibodies used in this study included rabbit anti-ferritin light chain (ab69090, Abcam), rabbit anti-KV1.3 (APC101, Alomone Labs), mouse anti-GAPDH (ab8245, Abcam), mouse anti-α-tubulin (B512, Sigma), anti-APP (ab133509, Abcam), rabbit anti-ADAM10 (Millipore), rabbit anti-BACE1 (Cell Signalling Technology), mouse anti-SIRT1 (1F3, Cell Signaling Technology), rabbit anti-insulin degrading enzyme (EPR6099, Abcam), rabbit anti-LC3 (ab48394, Abcam), rabbit anti-mTOR (PA1-518, ThermoFisher), rabbit anti-phospho-mTOR (S2448, ab109268, Abcam), rabbit anti-EIF2a (Cell Signalling Technology), rabbit anti-phospho-eIF2a (Ser51, Cell Signalling Technology), rabbit anti-NF-κB p65 (D14E12, Cell Signalling Technology), rabbit anti-acetyl-NF-κB p65 (Lys310, Cell Signalling Technology), rabbit anti-FMRP (ab17722, Abcam), and rabbit anti-SCAMP5 (ab3432, Abcam). Densitometric analysis was performed with ImageJ.

Iron Assay. The levels of total iron in human microglia were determined using a commercial assay (Abcam), following the manufacturer's instructions. Cellular iron content was determined from four confluent T25 flasks and normalized to total protein content determined by the Bradford assay.

Glutamate Assay. Glutamate levels in conditioned medium were assessed using a commercial kit according to the manufacturer's instructions (Sigma). Microglia were plated out at equivalent densities, and conditioned medium was generated as described above. After collection of the conditioned medium, the microglia were lysed, and protein concentration was measured using the Bradford assay. The glutamate concentrations were normalized to protein content in the lysates.

ADAM10 Activity Assay. The enzymatic activity of ADAM10 in SH-SY5Y cells was assessed using a commercial kit according to the manufacturer's instructions (Anaspec). Confluent SH-SY5Y cells were treated with SFM or conditioned medium from microglia for 24 h. The cells were lysed, and protein concentrations were measured using the Bradford assay. Equivalent amounts of protein were used in the ADAM10 activity assay to account for differences in cell number. Readings were taken at 520 nm every 5 min, and the initial rate was calculated for each condition.

Promoter Assay. The ADAM10 promoter construct was a gift and was as previously described.[102] SH-SY5Y cells were grown in 24-well plates seeded at 5×10^4 cells/well 24 h prior to transfection. Transfections of the promoter construct in pGL Basic (with firefly luciferase activity) were performed using FuGENE HD transfection reagent (Promega) as per manufacturer's instructions. To control for variation in transfection efficiency among replicates, promoter constructs were cotransfected with the Renilla luciferase vector, pRL-TK (Promega). At 24 h post-transfection, SH-SY5Y cells were harvested, and firefly and Renilla luciferase chemiluminescence were measured using the Dual-Luciferase Reporter Assay System (Promega) in a BMG FLUOstar Omega plate reader (BMG Labtech GmbH). Luciferase activity was calculated as the ratio of firefly to Renilla luciferase activity.

APP Cleavage Luciferase reporter Assay. The luciferase reporter assays was carried out as previously described.[103] SH-SY5Y cells were plated out at 5×10^4 cells/well in 24 well plates in triplicate for each condition and left to reattach for 18−24 h at 37 °C, 5% CO_2. Test wells were transfected with plasmids pFR-Luc (A firefly luciferase reporter construct pFR controlled by a synthetic promoter made of five tandem repeats of the yeast GAL4 activation sequence followed by a minimal TATA box), APP-Gal4 (pRC-CMV vector containing APP695 cDNA and a Gal4-DNA binding domain fused to its C-terminal end (cleavage of the AICD domain with Gal-4 domain attached leads to it binding to the pFR vector and expressing firefly luciferase) and pRL-TK (containing *Renilla* luciferase as above) using the transfection reagent Fugene HD. Control wells were transfected with pFR-Luc and pRL-TK only to assess background luciferase activity. The cells were transfected for approximately 24 h and then treated with SFM or microglial conditioned medium for 24 h. After lysing and harvesting, firefly luciferase and *Renilla* luciferase activity in the cells was measured with the Dual-Luciferase Reporter Assay System on a FLUOstar Omega plate reader. Luciferase activity was calculated as the ratio of firefly to *Renilla* luciferase activity.

Proliferation Assay. Proliferation of C8B4 microglia was assessed using the Abcam BrdU proliferation ELISA kit (Abcam, ab126556) according the manufacturer's instructions. Both untreated and iron-fed human microglia were plated at equal density ranging from 2000 to 20,000 cells per well in 96 well trays. The cells were exposed to BrdU (bromodeoxyuridine) for 24 h prior to starting the assay. Absorbance was measured at 450 nm in a FLUOstar Omega plate reader following the colorimetric assay.

***β*-Amyloid Assay.** Concentrations of β-amyloid (both 1−40 and 1−42) present in cell culture medium were determined using the Meso Scale Discovery (MSD) Sector Imager S 600 multiplex plate reader. The plate used for the analysis was the V-Plex Aβ Peptide Panel kit 1 (MSD). Medium was collected from SH-SY5Y cells that had been exposed either to conditioned medium or control medium (DMEM with B27 supplement) as described above and filtered through a 0.22 μm filter before applying to the plate without dilution. Concentrations were determined by comparison to a standard curve for each individual β-amyloid peptide. The values were adjusted for plating density by assessing the protein content of the cells used to generate the conditioned medium using the Bradford assay and dividing the β-amyloid concentration by the protein concentration.

Cytokine Assay. Cytokines secreted by the human microglia were also measured using the MSD Sector Imager S 600 multiplex plate reader. The cytokines were assessed using the V-PLEX Proinflammatory Panel 1 Human Kit (MSD). This kit measured IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, and TNF-α. Conditioned medium was collected from control and iron-fed microglia as described above. The microglia used to generate the conditioned medium were lysed and their protein content was measured using a Bradford Assay to account for differences in cell number.

Statistics. All statistical analyses were carried out in Microsoft Excel. Statistical analyses were conducted using a two-tailed Student's *t* test, setting statistical significance at *p*-value of <0.05. Data are expressed as the mean ± standard error (SE).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.8b00334.

Effect of iron on β-amyloid in SH-SY5Y cell medium (PDF)

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Author Contributions

D.R.B. obtained the funding and devised the project. All other aspects of the work were performed by D.M.A. and D.R.B.

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Notes

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Altered Processing of Beta-amyloid in SH-SY5Y cells induced by Model Senescent Microglia

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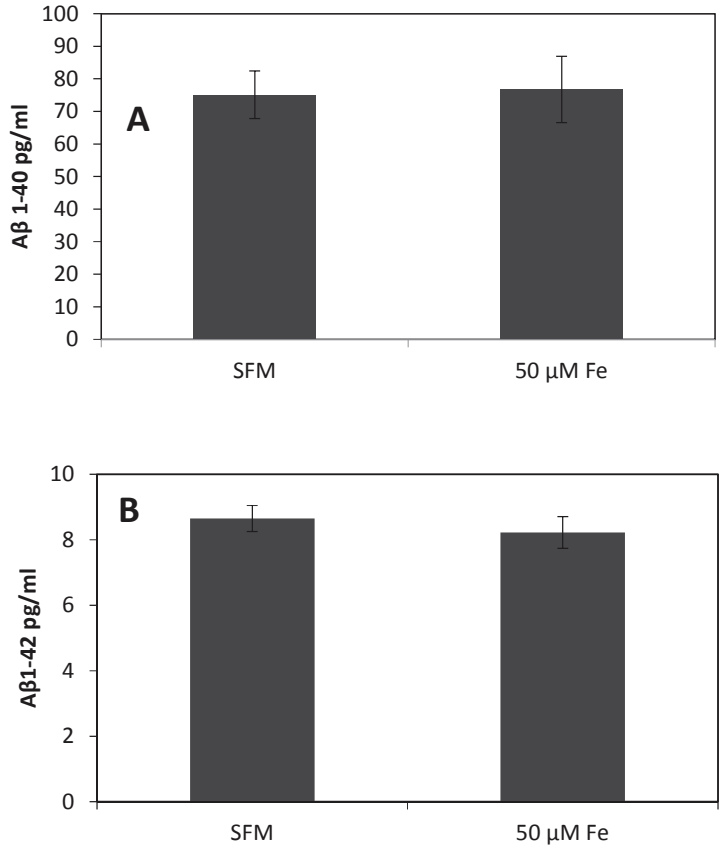
Supporting Information



Supplementary Figure 1 Effect of Iron on β-amyloid

SH-SY5Y cells were grown in serum free medium (SFM) for 24 h with or without the addition of 50 μM ferric ammonium citrate. After that time the medium from the cells and analysed for β-amyloid content using the MSD assay as described in the main paper. Both Aβ1-40 (A) and Aβ1-42 (B) were analysed. Exogenous iron had no significant effect ($p > 0.05$) on the levels of β-amyloid. Shown are the mean and s.e. for four independent experiments.

2.5 Concluding commentary

The work in the preceding chapter introduced a novel model of aging microglia and showed that a reduction in secreted β-amyloid degrading enzyme IDE by microglia can result in the accumulation of β-amyloid. The characterisation of the iron-fed microglial model included a measurement of L-ferritin levels as a measure of iron storage. The speed of storage or iron in the microglia was also measured by following the increase in ferritin expression over time presented in figure 2.1. The levels of ferritin measured after 1 day of supplementation were significantly elevated compared to untreated control and did not show further increases over the course of a month. This finding agreed with a previous report of iron storage by microglia that can occur very rapidly (Bishop et al. 2011). This suggests that any changes observed in the microglial phenotype after iron supplementation probably start occurring at a point after the cells have reached a saturation point in their iron storage capabilities.

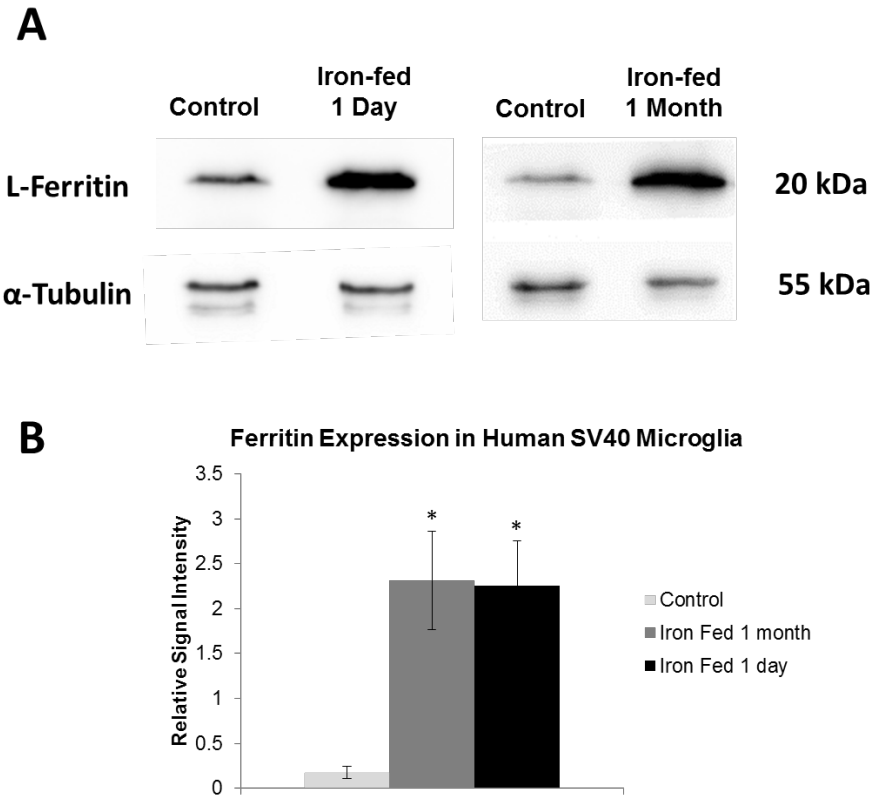


Figure 2.1. Increase in ferritin expression in iron-fed human SV40 microglia. **A.** Shows western blots for L-ferritin and α-tubulin of protein extracts of control microglia and microglia supplemented with iron for 1 day and 1 month. **B**. Shows densitometric analysis of western blots probed for L-Ferritin and α-tubulin with specific antibodies. B demonstrates the relative expression of L-ferritin compared to tubulin from protein extracts of control microglia, microglia supplemented with iron for 1 month and microglia supplemented with iron for 1 day. Data plotted are mean and SEM of 4 experiments. L-ferritin expression is significantly increased when iron is added to the cell culture medium but does not increase between 1 day and 1 month of supplementation. * indicates p-value≤0.05 compared to control.

The potential importance of this finding to the study of AD was discussed. While the previous chapter discussed the majority of our findings in regards these observations, we also pursued further lines of investigation in terms of the metabolism of APP. A pathway that may have affected β-amyloid production is changes in γ-secretase activity. The levels of the APP C-terminal fragments C89 and C99 were measured through western blotting in SHSY5Y cells treated with microglial conditioned medium. These levels can be used as an indicator of APP processing. Iron-fed microglia conditioned medium caused a significant increase in the levels of C-terminal fragments suggesting that APP cleavage and thus potentially β-amyloid production is also increased in the SHSY5Y cells. As APP cleavage by α- or β- secretase was not significantly increased, the activity of γ-secretase was measured using a luciferase reporter assay of Notch cleavage (another target of γ-secretase). Thus, luciferase expression was driven by the release of the Notch intracellular domain (NICD). SHSY5Y cells were treated with SV40 human microglial conditioned medium and Notch cleavage was measured (Figure 2.2). Notch cleavage, just as APP cleavage shown in Fig. 6A was significantly reduced by the presence of conditioned medium. However, iron-fed microglia conditioned medium induced a significant increase of Notch cleavage compared to control indicating that γ-secretase activity was also increased. This increase in γ-secretase activity could also play a role in the increased β-amyloid levels seen in Fig. 7.

The significance of the data presented in Figure 2.2 is that microglial conditioned medium could have a double effect on β-amyloid levels with both production and degradation being affected. The exact process through which β-amyloid production could increase under the effect of iron-fed microglia has not been determined. β-amyloid deposition has been shown to activate microglia resulting in the release of inflammatory cytokines. Previous reports have linked increased inflammation in the nervous system with increased β-amyloid deposition which creates a feedback loop (W.-Y. Wang et al. 2015; Sastre et al. 2008) γ-secretase mediated cleavage of APP has been reported to be affected by inflammatory cytokines such as TNF-α, IL-1β and IFNγ (Liao et al. 2004). It is possible that elevated levels of IL-1β in the iron-fed microglia conditioned medium could have resulted in the observed increase in γ-secretase activity.

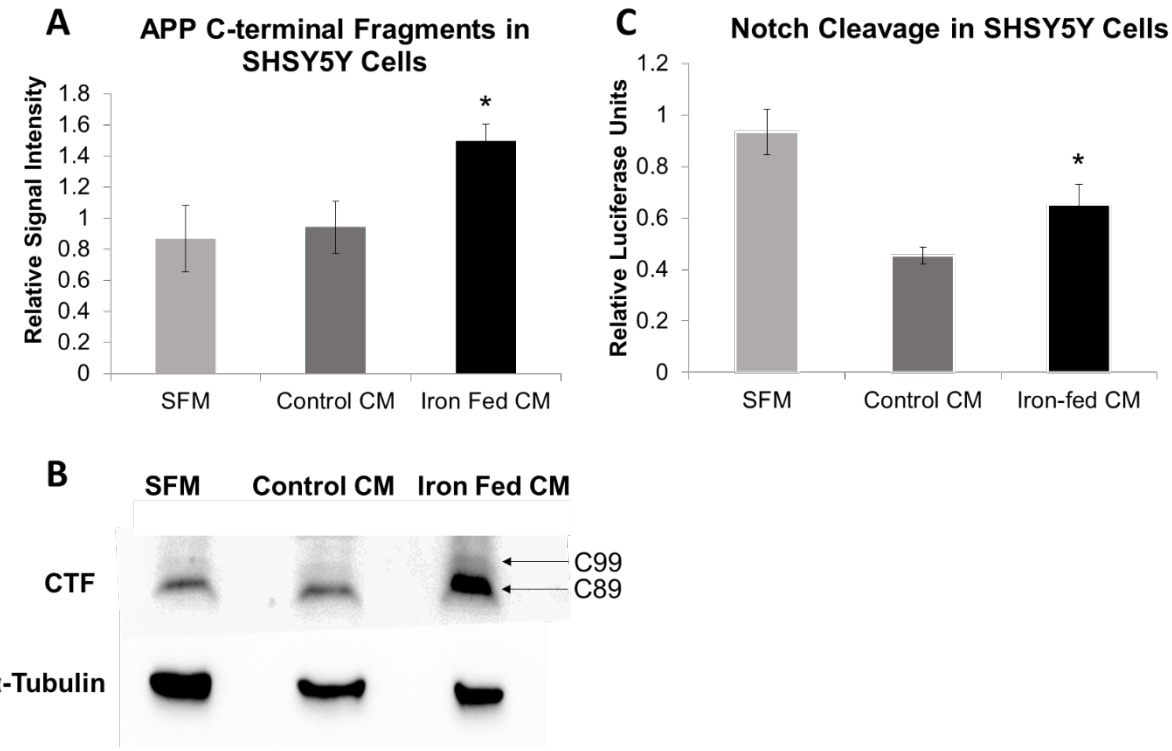


Figure 2.2. Altered γ-secretase APP processing in SHSY5Y cells treated with microglia conditioned medium. A. Conditioned medium was generated from human microglial cell line and was applied to SHSY5Y cells for 24h. SFM – serum-free medium control. Control – conditioned medium from untreated microglia. Iron-fed – conditioned medium from iron-fed microglia. The cells were lysed, and equal amount of protein were loaded onto a PAGE gel. The gel used was a 4% Tris-tricine stacking and 16% Tris-Tricine separating gel and the lysates were electrophoresed at 35mA/80V for 15 minutes and then 35mA/100V for 8 hours as described in (Schagger 2006). APP C-terminal fragments were measured with western blotting and the signal was normalised to tubulin expression. The data were analysed densitometrically. The data displayed are mean relative signal intensity and S.E.M. of four experiments. **B.** Representative western blots for APP CTFs. C99 and C89 and α-tubulin. **C.** SHSY5Y cells were transfected with a luciferase reporter plasmid that is activated by the release of the Notch intracytoplasmic domain post γ-secretase cleavage together with PRL-TK and PFR-Luc. The Notch cleavage luciferase reporter (Notch-Gal4), a pSecTag2 vector containing cDNA encoding for human Notch3 fused in frame at its C terminus to the yeast transcription factor Gal4 was provided by Dr. Robert J. Williams (Department of Biology & Biochemistry, University of Bath), as described in (Cox et al. 2015). After 24h they were treated with microglial conditioned medium from SV40 human microglia or SFM. On the following day the cells were lysed, and luciferase activity was measured using a Dual-Glo luciferase assay system (Promega) on a FLUOstar Omega Microplate Reader (BMG LABTECH) using the manufacturer's instructions.

Finally, Figure 2.3 is an alternative version of figure 9B that shows the entire gel for a western blot for eIF2a and illustrates that the ratio of p-eIF2a to total eIF2a is elevated in iron-fed microglia suggesting an increase in ER stress.

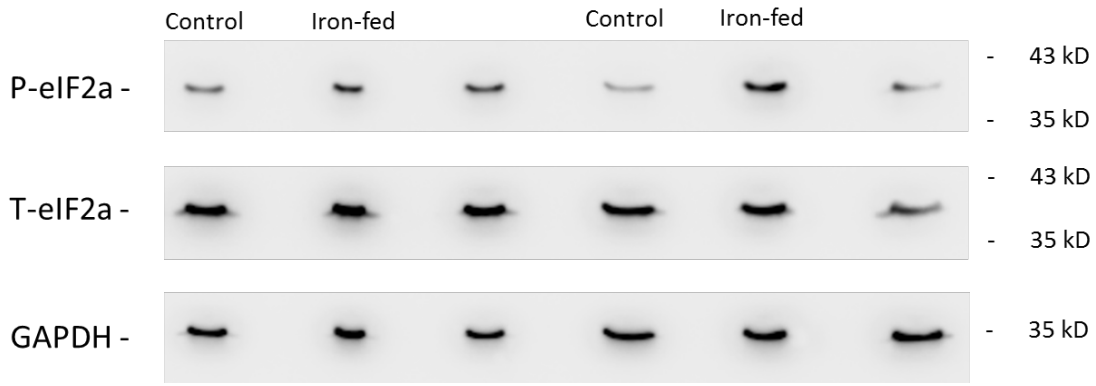


Figure 2.3. Expression of eIF2a in control and iron-fed human SV40 microglia. Protein extracts were prepared from control and iron-fed microglia. Western blotting and immunodetection were used to assess the level of p-eIF2a, total eIF2a and GAPDH.

Overall, the finding of this paper is useful in the context of establishing a simple and easy to use model of aged microglia and show that it can be used to replicate changes seen in AD.

3. Model Senescent Microglia Induce Disease Related Changes in α-Synuclein Expression and Activity

3.1 Introductory commentary

Aged microglia are thought to play a role in synucleinopathies, but the mechanisms by which they could influence α-synuclein expression, metabolism and aggregation have not been well investigated. In this paper we apply our cell culture-based aged microglia model to study this question.

We aim to characterise both a mouse microglial cell line and primary mouse microglia incubated in high levels of iron as a model for aged microglia. Our second aim was to show whether the aged microglia model can affect α-synuclein levels in SHSY5Y neuronal cells and whether that results in changes in α-synuclein aggregation. The focus of this paper was to document the molecular changes that occurred in the cells and to build a potential pathway through which α-synuclein could be perturbed.

The primary microglial cells and the mouse microglial cell line were characterised in a similar way as the human microglial cell line in Chapter 2. Their conditioned medium was used to induce effects on SHSY5Y cells. Changes to SH-SY5Y cells were investigated by measuring levels of α-synuclein with western blotting, α-synuclein promoter activity with a luciferase reporter assay, levels of α-synuclein enzymatic activity with a ferrireductase assay and levels of tetramer and aggregate formation by western blot. This multifaceted approach allowed us to conclude whether the effects of microglial conditioned medium on α-synuclein replicated ones seen in PD and other synucleinopathies.

The mouse microglial cell line C8B4 was chosen for the same considerations as in Chapter 2, namely that it allowed the continued generation of large numbers of microglia and that it expressed microglial markers. Primary microglia were harvested from mouse neonates, allowing a better yield and a longer lifespan in culture as compared to microglia from adult animals. The use of primary microglia was included to verify that the changes we observed were not as a result of using an immortalised cell line. However, primary microglia could not be used for all experiments as the yield was not sufficient for so many experiments.

3.2 Statement of contribution

This declaration concerns the article entitled:									
Model Senescent Microglia Induce Disease Related Changes in α-Synuclein Expression and Activity									
Publication status (tick one)									
draft manuscript		**Submitted**		**In review**		**Accepted**		**Published**	X
Publication details (reference)	Angelova, D & Brown, D 2018, 'Model Senescent Microglia Induce Disease Related Changes in Alpha-synuclein Expression and Activity' Biomolecules, vol. 8, no. 3, 67. DOI: 10.3390/biom8030067								
Candidate's contribution to the paper (detailed, and also given as a percentage).	The candidate contributed to/ considerably contributed to/predominantly executed the… Formulation of ideas: Researched and identified targets to pursue to take the project further once the initial effect was observed. (60%) Design of methodology: Modification of existing cell culture, western blotting and kit assay protocols to obtain quality data. (75%) Experimental work: Generated the data presented in figures 1,2,3,4,6,7,8,9,10 and table 1. (75%) Presentation of data in journal format: Preparation of figures and tables and proof reading of the manuscript. (50%)								
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.								
Signed	Dafina Angelova					**Date**	18.09.2018		

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3.4 Model Senescent Microglia Induce Disease Related Changes in α-Synuclein Expression and Activity

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Model Senescent Microglia Induce Disease Related Changes in α-Synuclein Expression and Activity

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Abstract: Aging is the most prominent risk factor for most neurodegenerative diseases. However, incorporating aging-related changes into models of neurodegeneration rarely occurs. One of the significant changes that occurs in the brain as we age is the shift in phenotype of the resident microglia population to one less able to respond to deleterious changes in the brain. These microglia are termed dystrophic microglia. In order to better model neurodegenerative diseases, we have developed a method to convert microglia into a senescent phenotype in vitro. Mouse microglia grown in high iron concentrations showed many characteristics of dystrophic microglia including, increased iron storage, increased expression of proteins, such as ferritin and the potassium channel, Kv1.3, increased reactive oxygen species production and cytokine release. We have applied this new model to the study of α-synuclein, a protein that is closely associated with a number of neurodegenerative diseases. We have shown that conditioned medium from our model dystrophic microglia increases α-synuclein transcription and expression via tumor necrosis factor alpha (TNFα) and mediated through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The conditioned medium also decreases the formation of α-synuclein tetramers, associated ferrireductase activity, and increases aggregates of α-synuclein. The results suggest that we have developed an interesting new model of aged microglia and that factors, including TNFα released from dystrophic microglia could have a significant influence on the pathogenesis of α-synuclein related diseases.

Keywords: synuclein; tumor necrosis factor alpha; microglia; aging; iron; cytokines; tetramer

1. Introduction

The study of neurodegenerative diseases has become a major incentive. This is due to their increased prevalence as the human population lives longer. This statement in itself immediately implies that a clearer understanding of these diseases will only come by greater insight into how the prevalence of these diseases is related to the aging process in the brain. Aging of the human brain is commonly associated with cognitive decline and it is the primary risk factor for the development of Alzheimer's disease (AD) [1]. Despite these facts, the molecular biology of the aging brain has not been studied extensively. Some of the factors that change as we age include: the environment the neurons are exposed to and their transcriptome.

An example of the changing environment in the aging brain is the changes in the supporting cells in the brain, including microglia. Healthy microglia monitor their environment, phagocytosing debris, and releasing numerous molecules that can impact other cells [2]. They include hydrogen peroxide (H_2O_2), nitric oxide (NO), inflammatory cytokines, proteases, and neurotransmitters. Activated microglia can act as antigen presenting cells and activate T-cells. After an infection has been dealt with microglia can recruit cells that are involved in neuronal repair and secrete anti-inflammatory cytokines [3]. The idea of aging microglia stems from histological observations of healthy aged brains where the cells often develop dystrophic phenotypic characteristics [4].

Resting microglia have a ramified morphology with many fine processes extending from the cell body. Dystrophic microglia found in aging brains lose this fine process ramification. Dystrophic microglia often develop abnormally shaped processes with spheroidal swellings and cytoplasmic fragmentation (cytorrhexis) [5]. Dystrophic microglia have also been associated with the increased release of toxic ROS (reactive oxygen species) and inflammatory cytokines and impaired phagocytic ability [6–8]. The proinflammatory cytokines found to be released by dystrophic microglia include IL-6 (interleukin-6), TNF-α (tumor necrosis factor alpha), and IL-1β (interleukin 1-beta) [7]. However, one of the most unique changes observed in dystrophic microglia in the aging brain is the very high accumulation of iron, which is found to be stored in proteins, such as ferritin [9–11].

The presence of healthy glial cells is critically important to neuronal wellbeing. Microglia maintain homeostasis in the healthy brain and fight infection, when it is present, through a complicated system of signalling molecules [12]. The importance of microglia to neurons is supported by higher incidence of dystrophic microglia and microglial apoptosis in Alzheimer's disease [13]. The inflammation of the nervous system in neurodegenerative disease was thought to be due to activated microglia. However, low, but sustained, release of inflammatory factors and impaired neuroprotective ability of microglia seen in neurodegeneration could be due to dystrophic changes instead [14].

The cytosolic protein alpha-synuclein (α-syn) is associated with a range of neurodegenerative diseases, including Parkinson's disease (PD) Dementia with Lewy bodies (DLB), the Lewy body variant of Alzheimer's disease, and multiple system atrophy. Aggregated α-syn is concentrated in structures termed Lewy bodies and Lewy neurites that are associated with the synucleinopathies [15,16]. Extracellular α-syn is present as aggregates in both the substantia nigra of PD patients [16] and senile plaques of AD brains in the form of the non-Aβ component (NAC) [17,18]. Clear links between α-syn and neurodegeneration have been found. Neuronal cell loss and Lewy body-like inclusions occur in animal models overexpressing α-syn [19] and the rescue of dopaminergic cells from death occurs following the down-regulation of α-syn expression in the substantia nigra of a Parkinson's disease rat model [20].

In PD there has been discussion of the possible involvement of microglia [21] and experiments with rodent PD models have shown that microglial activation can cause PD-like symptoms [22]. Parkinsonian changes in primate brains induced by manganese have been shown to be accompanied by dystrophic changes in microglia [23]. Additionally, dystrophic microglia have been identified in DLB [24]. Age-related changes in microglia have also been suggested to play a role in Parkinson's disease [25].

In the current work, we establish iron overload as a mechanism to switch microglial phenotype to one that has many of the characteristics of senescent microglia. Iron overload was achieved by growing microglia in high concentrations of iron. We also show that iron overloaded (iron-fed) microglia release factors, including increased levels of the cytokine TNFα that caused an increased expression of α-syn, altered its activity, and increased its aggregation. We argue that such a model of senescent microglia could be utilized to improve models for the study of neurodegenerative diseases by the incorporation of this age-related change.

2. Materials and Methods

Reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.1. Cell Culture

SH-SY5Y (human neuroblastoma) cells were cultured in 45% DMEM (Dulbecco's modified Eagle's medium)/45% Ham's F12 (LONZA, Basel, Switzerland) supplemented with 10% FBS (foetal bovine serum) (Labtech, Heathfield, UK), and 1% penicillin/streptomycin. Cells were maintained at $5 \times 10^6/75$ cm^2 at 37 °C and 5% CO_2 in a humidified incubator. The neuronal status of SH-SY5Y cells was monitored by reverse transcription polymerase chain reaction (RT-PCR) with primers for tyrosine hydroxylase (TH), dopamine transporter (DAT), and vesicle monoamine transporter 2 (VMAT2).

Cell lines derived from SH-SY5Y cells and overexpressing α-syn were developed by stable transfection of plasmids (pCDNA3.1) containing the open reading frame (ORF) of the protein using Fugene (Promega, Southampton, UK). The cell line generated was as previously described [26].

The microglial cell line used in this study was C8B4 (CRL2540, American Type Culture Collections (ATCC), mouse). The cell line was grown in DMEM with 10% FBS, and 1% penicillin/streptomycin. Primary microglial cells were also used for some experiments and were prepared, as previously described [27,28]. Mouse primary microglia were generated from new born mice (balb/c). Mice were sacrificed according to a schedule 1 procedure. The procedure was endorsed by the Animal Welfare and Ethics Review Board of the University of Bath and was in accordance with the Guidance on the operation of the Animals (Scientific Procedures) Act 1986. Brains of the mice were dissected and dissociated by both trypsinization and mild mechanical disruption. Cultures were seeded with cells from 4–5 brains per 75 cm^2 flask. Cells were grown at 37 °C and 5% CO_2 for two weeks in the same medium as the microglial cell lines. The resultant cultures were mixed glial cultures composed of predominantly astrocytes and microglia. Microglia were separated from other glia by partial trypsinization, which causes the detachment of the overlying astrocytes [28]. The remaining cells were then collected by further trypsinization and panning. Microglia have higher adhesiveness and most remaining contaminating cells could be removed after 20 min of plating. The remaining adherent cells were microglia. Routine verification of purity was determined either by phenotype or by staining with ferritin.

Microglia were cultured in high iron to induce a dystrophic/senescent phenotype. Microglial cell lines were grown in medium containing in 500 μM ferric ammonium citrate for a minimum of two weeks. Ferric ammonium citrate was prepared in deionized water at 25 mM and filtered (0.22 μm filter) before addition to cultures. Cell lines were maintained under these conditions until being used for experiments. Primary microglia were treated with 500 μM ferric ammonium citrate while in mixed glial cultures before purification. Prior to experiments control and iron-fed primary microglia were isolated from the mixed cultures and plated at 30% density in six well trays. Conditioned medium was generated from microglial cell lines and primary microglia plated at 30% density. The medium used for the generation of conditioned medium was DMEM supplemented with B27 without antioxidants (Gibco, ThermoFisher, Waltham, MA, USA) and 1% pen/strep. Iron-fed microglia were extensively washed to remove excess iron before medium was applied. Conditioned medium was collected after 48 h, centrifuged at 170× *g* to remove debris, and filtered with a 0.45 μm filter before use in experiments. The cell density that was used to generate conditioned medium was confirmed by lysing the cells after collecting the conditioned medium and measuring the protein concentration using a Bradford assay (Bio-Rad, Watford, UK). The data was normalized to this value.

2.2. Western Blotting

Cells were lysed in PBS with 0.5% Igepal CA-630 and 'complete' protease inhibitor cocktail (Roche, Welwyn Garden City, UK), sonicated 20 s on ice, incubated on ice for 20 min, and centrifuged 10,000× *g* for 5 min to remove insoluble membranes. Protein concentration was determined with a Bradford protein assay (Bio-Rad), according to the manufacturer's instructions. Protein concentrations were normalized and the samples were boiled for 5 min with 1× Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. Samples were loaded into either a 10% or a 14% (depending on molecular weight of the protein) acrylamide SDS-PAGE gel, with a buffer of Tris (250 mM) + glycine (1.92 M) + sodium dodecyl sulfate (SDS) (0.1% *w*/*v*), run at 250 V/35 mA for 45–60 min. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by a semi-dry transfer apparatus, run at 25 V/100 mA for 1.5 h. Membranes were blocked in 5% *w*/*v* non-fat milk powder in TBS-T (Tris-buffered saline + 0.05% tween 20) for one hour, incubated with primary antibody for 1–2 h or overnight, and washed 3 × 15 min in TBS-T. Membranes were blocked again and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. A further 3 × 15 min washes were performed, and the membranes developed with

Luminata Crescendo or Luminata Forte ECL substrate (Millipore, Watford, UK) and imaged with a Fusion SLCCD imaging system (Vilber Lourmat, Collégien, France).

Rabbit monoclonal anti-α-synuclein (MJFR1, Abcam, Cambridge, UK) was used for human α-synuclein detection at a dilution of 1:4000. Mouse monoclonal anti-α-tubulin (T5186, Sigma) was used at a dilution of 1:10,000. Anti-L-ferritin mouse monoclonal (SC-25616, Santa Cruz, Dallas, TX, USA) was used at 1:5000, anti-Kv1.3 rabbit polyclonal was used at 1:400 (APC101, Alomone, Jerusalem, Israel), and anti- Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal was used at 1:2000 (6C5, Abcam). Densitometry was carried out using ImageJ.

2.3. Detection of α-Synuclein Tetramers and Aggregates and Ferriductase Assay

Measurement of ferrireductase activity was performed on extracts of SH-SY5Y cells transfected to overexpress human α-syn. The stable cell line and the method to measure the activity were as previously reported [29]. Tetramers were detected by crosslinking the extracts with the crosslinker, disuccinimidyl suberate (DSS) (Sigma), according to the manufacturer's instructions and as previously described [29]. Crosslinked species were detected with western blot, as described above, for non-crosslinked α-syn. High molecular weight aggregates of α-syn were detected, as previously described in cross linked samples of SH-SY5Y cells overexpressing α-syn [26].

2.4. Iron Assay

The levels of total iron in C8B4 cells and conditioned medium were determined using a commercial assay (Abcam) and following the manufacturer's instructions. Cellular iron content was determined from four confluent T25 flasks and normalized to total protein content determined by the Bradford assay. Conditioned medium was concentrated 10-fold using a Speedvac Concentrator (Savant, ThermoFisher).

2.5. Reactive Oxygen Species Assay

The method to determine reactive oxygen species was based on Uy et al., 2011 [30]. Microglia (both control and iron-fed) were re-plated to equivalent density. The medium of the cells was replaced with serum free medium (DMEM) supplemented with B27 without antioxidants. After 24 h, the medium was collected from the microglia and then cleared by centrifugation at 10,000× *g* for one min. Control medium (50 µL) was used as a blank. The assay was executed in a white 96 well plate. 10, 20 30, 40, and 50 µL of the conditioned medium was plated into the plate and made up to 100 µL with distilled water. The assay was initiated by the addition of 100 µL of chemiluminescence reagent (Luminata Crescendo, Millipore). The plate was incubated in the dark for five min before reading. Measurements of luminescence were performed using the Omega FLUOstar plate reader (MBG, Labtech GmbH, Offenburg, Germany) at 482 nm. Background was subtracted for all values.

2.6. Cytokine Quantitation

Analysis of cytokines present in conditioned medium was determined while using the Meso Scale Discovery (MSD, Rockville, MD, USA) Sector S 600 multiplex imager system. The plate used for the analysis was the V-Plex Proinflammatory mouse kit 1 (MSD). This kit allowed for the assessment of the following cytokines: TNF-α, IFN-γ (interferon gamma), IL-1β, IL-2, IL-4, IL-5, IL6 (interleukin-6), IL-10 (interleukin-10), IL-12p70 (interleukin-12p70), and KC/GRO (keratinocyte chemoattractant/growth-regulated oncogene). Conditioned medium was prepared as described above and filtered through a 0.22 µm filter before applying to the plate as per the manufacturer's instructions. Concentrations were determined by comparison to a standard curve for each individual cytokine. The values were adjusted for plating density by assessing the protein content of the cells used to generate the conditioned medium while using the Bradford assay and dividing the cytokine concentration by the protein concentration.

2.7. Promoter Assays

SNCA Promoter fragments and assay conditions were as previously described [31]. SH-SY5Y cells were performed in 24-well plates seeded at 5×10^4 cells/well 24 h prior to transfection. Transfections of promoter constructs in pGL Basic (with firefly luciferase activity) were performed using FuGENE HD transfection reagent (Promega), as per manufacturer's instructions. To control for variation in transfection efficiency among replicates, promoter constructs were co-transfected with the Renilla luciferase vector, pRL-TK (Promega). At 24 h post transfection, SH-SY5Y cells were harvested and firefly and Renilla luciferase chemiluminescence were measured while using the Dual-Luciferase Reporter Assay System (Promega) in a BMG FLUOstar Omega plate reader (BMG Labtech GmbH, Offenburg, Germany). Luciferase activity was calculated as the ratio of firefly to Renilla luciferase activity.

2.8. Proliferation Assay

Proliferation of C8B4 microglia was assessed using the Abcam BrdU proliferation ELISA kit (ab126556, Abcam) according the manufacturer's instructions. Both untreated and iron-fed C8B4 microglia were plated at equal density ranging from 2000 to 20,000 cells per well in 96-well trays. The cells were exposed to BrdU (bromodeoxyuridine) for 24 h prior to starting the assay. Absorbance was measured at 450 nm following the colorimetric assay.

2.9. Toxicity Assay

The level of cell death during treatment of C8B4 cells with 500 µM ferric ammonium citrate was measured using the Roche Cell Death Detection ELISA^{PLUS} kit. The procedure was carried out following the manufacturer's instructions. C8B4 cells were plated into 24 well trays at low density (10%). The treatment was carried out over ten days (medium changed every two days). Measurements of cell death were carried at 1, 2, 4, 6, 8, and 10 days of treatment. The level of cell death in iron-fed cells was compared to that of untreated cells as a percentage.

2.10. Statistics

All of the statistics were carried out in Microsoft Excel. Statistical analyses were conducted using a two-tailed Student's *t* test, with statistical significance at *p*-value of <0.05. Data are expressed as the mean $\pm$ standard error (S.E.M.).

3. Results

3.1. Generation and Characterisation of a Senescent Phenotype in Microglia

We hypothesized that the senescent microglial phenotype might be related to changes in iron storage. Iron (particularly Fe(II)) causes damage to macromolecules [32] and simply increasing the levels of iron that is stored may be sufficient to induce changes seen in the aged phenotype. For this reason, we grew the murine microglial cell line C8B4 in 500 µm ferric ammonium citrate for at least two weeks. The C8B4 cell line was used because it allowed for the production of large numbers of microglia to facilitate the study. C8B4 cells grown under these conditions will be referred to as iron-fed microglia.

Iron-fed C8B4 microglia showed morphological changes that are similar to dystrophic microglia. Under normal culture conditions (Figure 1A) C8B4 cells showed a small cell body with multiple projections and frequent ramification. In contrast, iron-fed microglia (Figure 1B) showed no ramification and little to no projections and frequent membrane fragmentation. We also examined primary microglia for evidence of similar phenotypic changes. While primary microglia under normal culture conditions showed projections (Figure 1C), iron-fed primary microglia were largely ameboid with no projections at all (Figure 1D).

After two weeks of treatment, iron-fed microglia were analysed for changes in both iron content and the iron storage protein ferritin. Cells were washed free of serum and iron and left for 24 h in

serum free medium before harvesting in serum free medium. Analysis of the iron content of C8B4 microglia showed that there was an approximate five-fold increase in the amount of iron that is present in the cells when iron-fed (Figure 2A). Conditioned medium generated from iron-fed microglia showed no significant increase in the amount of iron released (Figure 2B). Also, the level of iron released/present in the medium conditioned by the microglia was lower than the amount if iron present in the medium that these cells were normally cultured in (with 10% serum). Iron is mostly stored within the protein ferritin in microglia and increased ferritin expression is associated with a change to a dystrophic phenotype [9]. We assessed the level of L-ferritin in iron-fed C8B4 by western blot (Figure 2C,D). Iron-fed microglia showed a very high (five-fold) increase in the level of ferritin expressed. These results show that iron-fed microglia have a similar pattern of increased iron storage as dystrophic microglia.

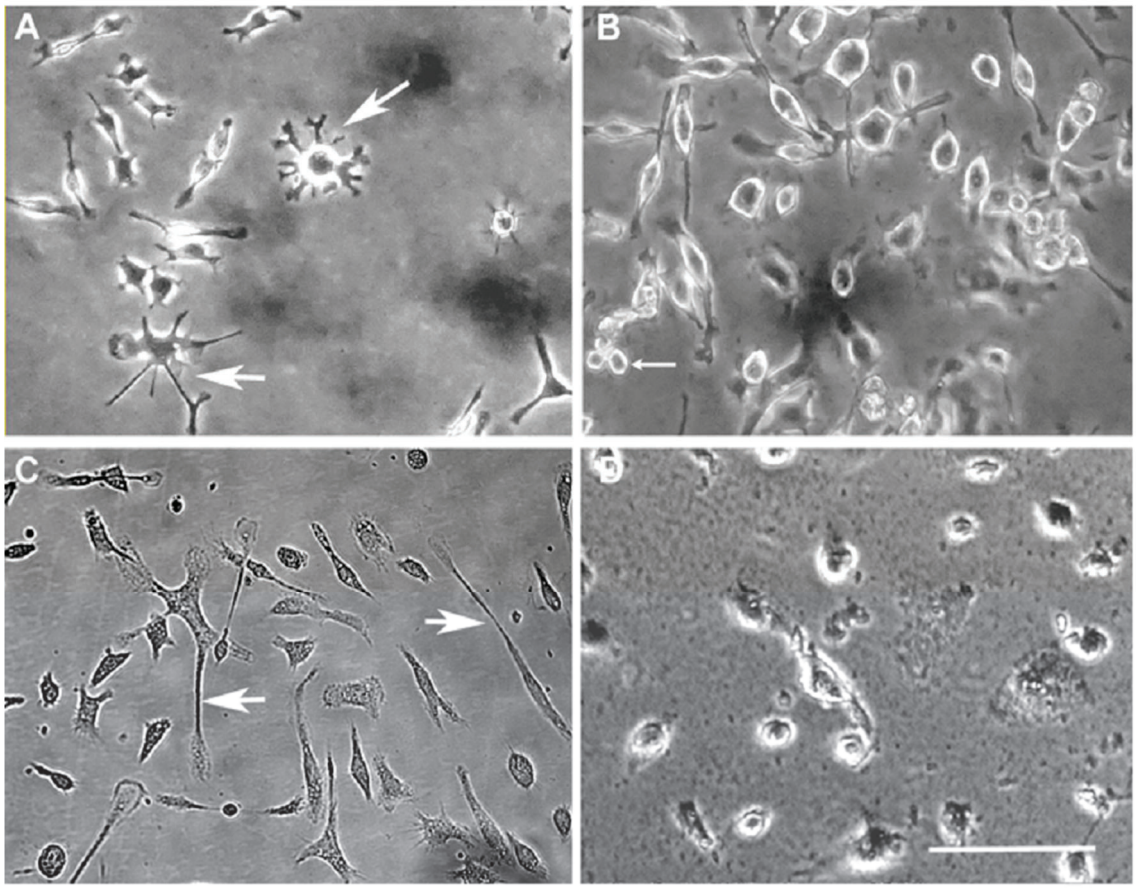

Figure 1. Phenotype of iron-fed microglia. Photomicrographs of microglia were prepared from both C8B4 (**A**,**B**) and primary mouse microglia (**C**,**D**) in culture. The microglia were either grown in control conditions (**A**,**C**) or grown in 500 μM ferric ammonium citrate (iron-fed, **B**,**D**). Both C8B4 and primary microglia showed clear phenotypic changes when iron-fed. These include loss of the projections seen in control cells (large arrow head) and the appearance of cytoplasmic fragmentation (small arrow). Scale bare = 50 μm.

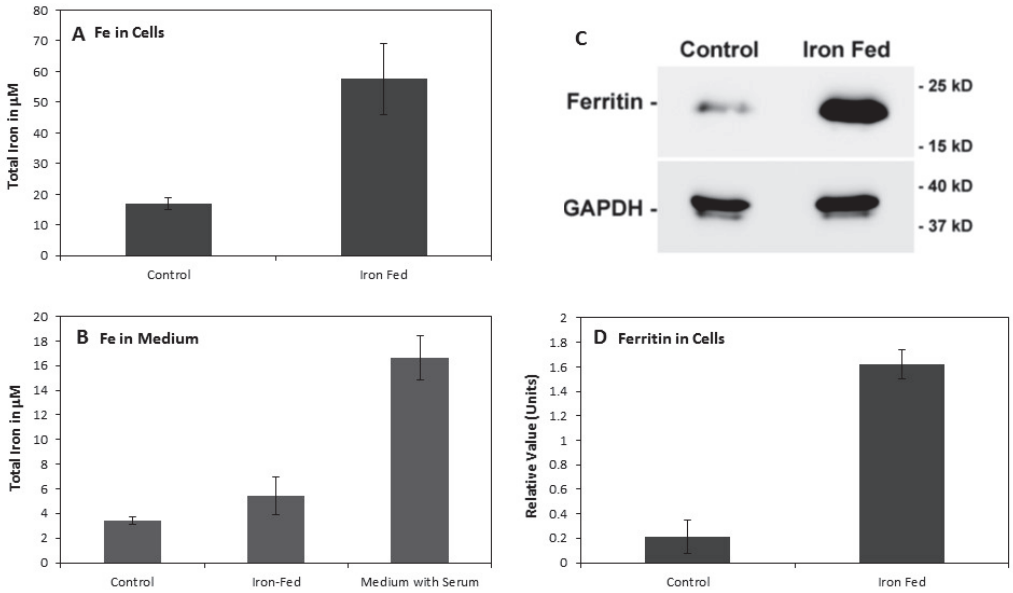

Figure 2. Changes in iron storage C8B4 microglia that had been grown in the presence of high ferric ammonium citrate were analysed for changes in iron content, iron release, and ferritin expression. (**A**) We used a commercial kit to analyse the iron content of microglia grown in 500 µM iron for at least two weeks. Microglia were washed three times before analysis to remove excess iron from the medium. Extracts were then made from the microglia and the iron content determined for both control and iron-fed microglia. The analysis showed a high and significant ($p < 0.05$) change in the iron within C8B4. (**B**) The level of iron released into the culture medium was assessed similarly. Conditioned medium (B27 supplemented DMEM was produced from control and iron-fed C8B4 microglia by exposing them for 24 h. The medium was concentrated 10-fold by lyophilisation and the concentration compared to serum containing medium used to maintain the C8B4 microglia. While iron-fed microglia showed no significant increase in iron released in the medium compared to controls ($p > 0.05$) this concentration was significantly lower than found in normal serum supplemented conditioned medium ($p < 0.05$). (**C**) The levels of L-ferritin were determined in iron-fed microglia by western blot. Protein extracts were prepared and equal amounts of protein from control and iron-fed microglia were run on a 10% PAGE gel, transferred to a membrane and L-ferritin was detected on the membrane with a specific antibody. Another antibody was used to detect GAPDH to confirm equivalent protein loading. (**D**) The densitometric quantitation following normalization to GAPDH levels showed there was a major and significant ($p < 0.05$) increase in L-ferritin expression levels in iron-fed microglia. Shown are the mean and S.E.M. of four experiments for each part.

3.2. Proliferation and Cell Death

We determined the impact of the iron-fed phenotype on the rate of proliferation of C8B4 microglia. We used an ELISA assay based on BrdU (bromodeoxyuridine) incorporation. Both control and iron-fed microglia were plated at a range of densities in serum free medium supplemented with B27 and exposed to BrdU overnight. The level of incorporation of the label was then assessed according to the manufacturer's instructions. The level of BrdU incorporation was significantly different between iron-fed and control microglia at all plating densities except for the highest (Figure 3A). At the highest density, cellular crowding probably impacted the rate of proliferation. However, the results suggest that iron-fed microglia proliferate at a significantly lower rate than control microglia.

We also wished to determine if C8B4 microglia experience cell death during the treatment that results in the iron-fed phenotype. C8B4 microglia were exposed to 500 µM ferric ammonium citrate for 10 days. Cell death was measured using an ELISA kit that assesses cytoplasmic histone-associated DNA fragments. The assessment was carried out at 1, 2, 4, 6, 8, and 10 days of treatment and compared to the

value that was obtained from the untreated control as a percentage. After one day of treatment there was no significant difference to the untreated control (Figure 3B). However, on all subsequent days the level of cell death detected fell significantly below that of the untreated control and the one-day treatment. After day 2, there was no significant change in the level of cell death. These results suggest that the treatment with iron had no significant toxic effect, and in fact there was a significant reduction in spontaneous cell death. However, it should be kept in mind that there was also a significant reduction in cell proliferation in iron-fed microglia, which would suggest that the difference observed is a result of reduced cell number rather than reduced spontaneous cell death.

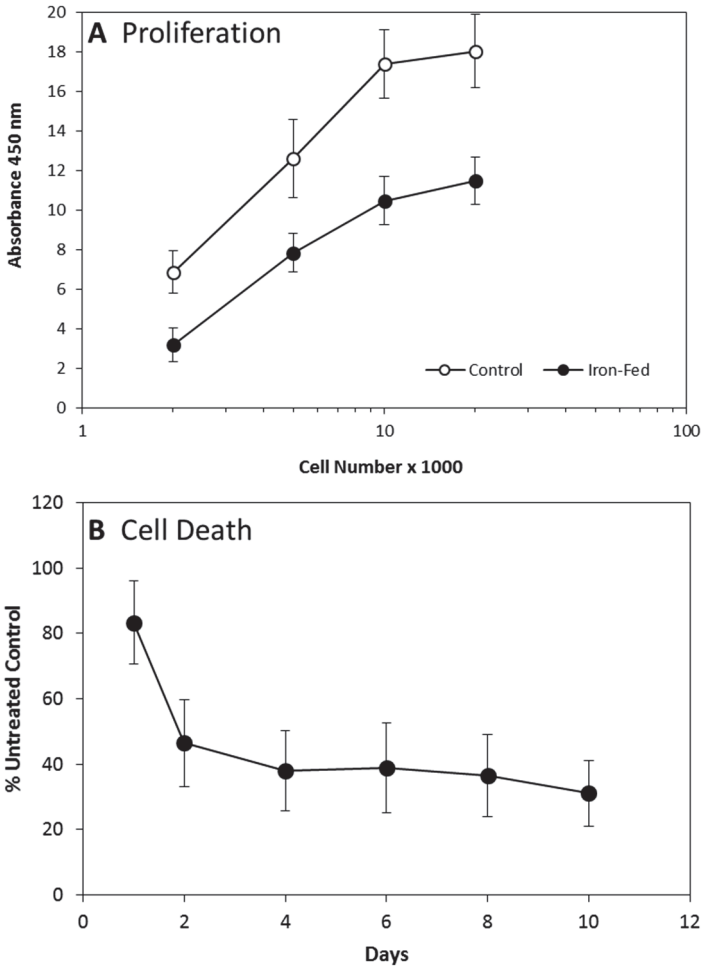

Figure 3. Proliferation and Cell Death (**A**) The rate of proliferation of C8B4 microglia was assessed using a bromodeoxyuridine (BrdU) based ELISA kit. Both control and iron-fed microglia were plated into a 96 well plate at a range of densities and grown overnight. BrdU was then added for a further 16 h before the ELISA assay was used to assess incorporation levels. The level of incorporation was assessed by a colorimetric assay with a read out at 450 nm. Iron-fed microglia showed significantly lower levels of proliferation at all plating densities other than the highest ($p < 0.05$). Shown are the mean and S.E.M. of four separate experiments. (**B**) The level of cell death in C8B4 cells was assessed during their initial treatment with 500 μM ferric ammonium citrate. C8B4 cells were plated in 24-well trays at low density (20% confluency). The cells were then treated with iron for up to 10 days. The level of cell death was assessed using a commercial ELISA kit that determines the levels of histone associated DNA fragments in the cytoplasmic fraction. The results showed a significantly lower level of cell death in cells treated with iron for 2–10 days ($p < 0.05$). Only cells treated for one day showed no significant difference to the untreated cells ($p > 0.05$). Shown are the mean and S.E.M. of four separate experiments.

3.3. Altered Protein Expression in Iron-Fed Microglia

The characterization of the senescent microglia phenotype by altered protein expression remains elusive, as there are currently no agreed changes that conclusively define microglia as being dystrophic. However, a recent study suggested that aged microglia show an increase in the potassium channel Kv1.3 [33]. We analysed the expression of Kv1.3 by iron-fed C8B4 using western blotting and found a significant increase relative to control cells (Figure 4). This suggests that similar to aged microglia, iron-fed microglia expressed increased levels of this potassium channel.

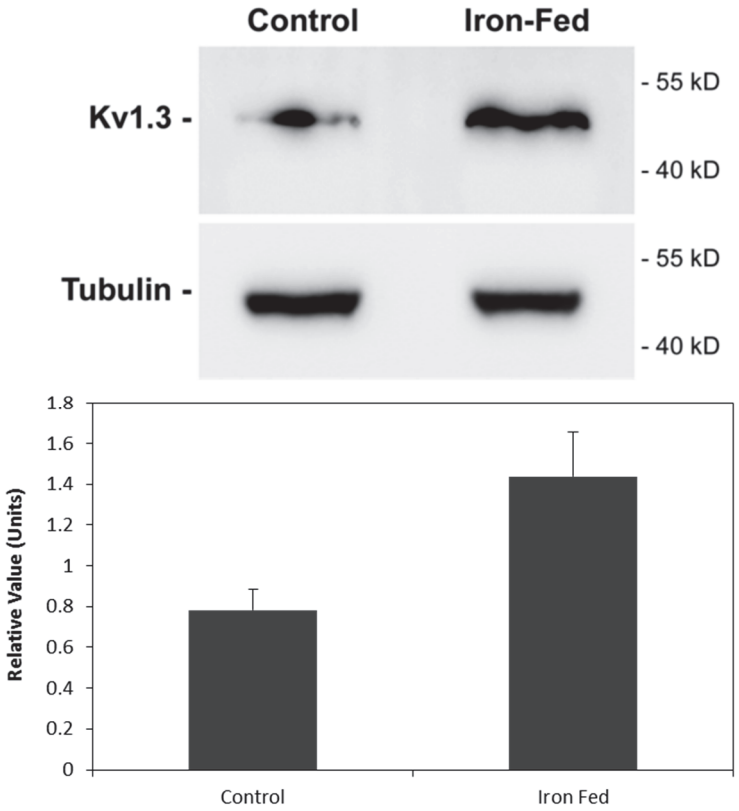

Figure 4. Expression of the potassium channel Kv1.3. Protein extracts were prepared from control and iron-fed C8B4 microglia. Western blot analysis was carried out to determine the level of Kv1.3 in the microglia. Bands for the protein were observed in both control and iron-fed microglia. Levels of tubulin were also determined to verify protein loading. The results showed a significant ($p < 0.05$) elevation of Kv1.3 in iron-fed microglia. Shown are the mean and S.E.M. of four separate experiments.

3.4. Reactive Oxygen Species Production

One of the most common changes in microglia is an increased production of (ROS)upon activation. We wished to assess whether iron-fed microglia also show a change in ROS production. We used a simple spectrophotometric assay to assess the change in ROS generated by iron-fed C8B4 microglia compared to control microglia. We generated conditioned medium from both kinds of microglia plated at an equal density by exposure of the microglia to serum free medium with the B27 antioxidant free supplement overnight. We applied increasing volumes of the medium to the assay and measured the ROS generated. Iron-fed microglia produced significantly higher levels of ROS than control microglia (Figure 5). This suggests that iron-fed microglia show some aspects of an activated phenotype similarly to aged microglia.

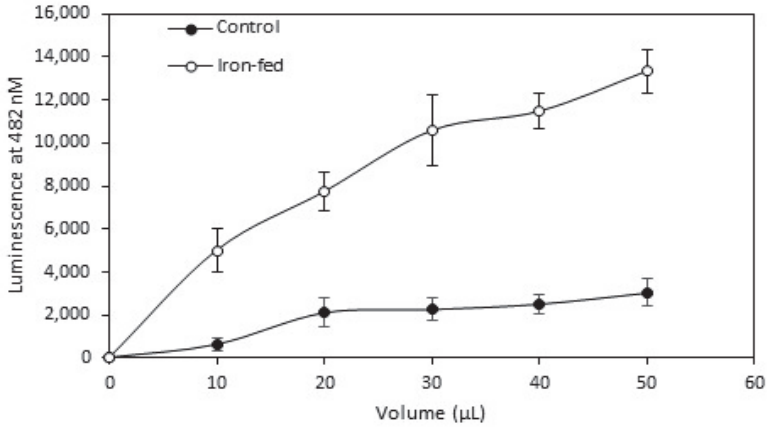

Figure 5. Reactive oxygen species (ROS). Control and iron-fed C8B4 microglia plated at equal density were exposed to serum free medium (without phenol red) for 24 h. Medium was collected and centrifuged and assessed for ROS using a luminescence assay with detection at 482 nm. Medium from iron-fed microglia showed significantly ($p < 0.05$) higher levels of ROS than control microglia. Shown are the mean and S.E.M. of five separate experiments.

3.5. Cytokine Expression

Cytokines produced by microglia constitute the single most significant way that microglia interact with other cells. Therefore, changes in cytokine expression would indicate a potential mechanism by which dystrophic microglia could influence neuronal activity. A sensitive assay system was used to measure the level of multiple proinflammatory cytokines produced by both C8B4 and primary mouse microglia in culture. We analysed 12 different cytokines secreted into conditioned medium. However, in both primary and C8B4 microglia a number of tested cytokines had expression levels below the detection limits of the assay (IFNγ, IL-2, IL-4, IL-5, IL-8, IL-12p70, and IL-13). KC/GRO was significantly elevated in iron fed primary microglia but could not be detected in C8B4 microglia (Table 1). IL-1β, IL-6, IL-10, and TNFα were detected in the conditioned medium of both cell types, but the changes that were observed in the two cell types were only the same for TNFα and IL-6. TNFα was significantly elevated for both cell types when iron-fed and IL-6 was significantly decreased. Iron-fed C8B4 cells also showed an elevated level of IL-1β, but decreased levels of IL-10. In contrast, iron-fed primary microglia showed decreased levels of IL-1β and increased levels of IL-10.

Table 1. Cytokine released into conditioned medium.

	C8B4 Microglia		Primary Mouse Microglia	
Cytokine (ng/mg)	**Control**	**Iron-Fed**	**Control**	**Iron-Fed**
TNFα	142.82 ± 12.40	192.46 ± 5.52 *	37.42 ± 3.74	84.19 ± 3.45 *
IFNγ	n.d.	n.d.	n.d.	n.d.
KC/GRO	n.d.	n.d.	698 ± 16	2113 ± 398 *
IL-1b	2.93 ± 0.27	3.83 ± 0.22 *	0.99 ± 0.08	0.34 ± 0.03 *
IL-2	n.d.	n.d.	n.d.	n.d.
IL-4	n.d.	n.d.	n.d.	n.d.
IL-5	n.d.	n.d.	n.d.	n.d.
IL-6	490.96 ± 49.77	430.77 ± 18.05	356.09 ±4 7.84	117.56 ± 24.22 *
IL-8	n.d.	n.d.	n.d.	n.d.
IL-10	6.20 ± 1.04	0.84 ± 0.26 *	5.00 ± 0.37	10.34 ± 0.98 *
IL-12p70	n.d.	n.d.	n.d.	n.d.
IL-13	n.d.	n.d.	n.d.	n.d.

Serum free conditioned medium was generated from cultured microglia over 24 h and the levels of cytokines assessed with the Meso Scale Discovery (MSD) ELISA system. Cytokine concentrations were determined by comparison to a standard curve for each cytokine. Values were the concentration in the medium (ng/mL) divided by the concentration of the protein in the cells that were used to generate the conditioned medium (mg/mL). Shown are the mean (ng/mg) and S.E.M. for five (C8B4) and three (primary) different experiments. * Indicates a significant difference between control and iron-fed ($p < 0.05$). n.d. indicates not detectible due to the levels detected being below the level of lowest standard on the standard curve for that cytokine.

3.6. Conditioned Medium from Iron-Fed Microglia Caused Increased α-Synuclein Expression

Changes in α-syn expression can easily be assessed using western blotting of protein extracts from cell lines, such as SH-SY5Y. Conditioned medium was generated from both control and iron-fed C8B4 microglia plated at equal density (40%). Iron-fed C8B4 cells had been grown in 500 μM ferric ammonium citrate for at least two weeks before use. The iron-fed cells were washed with fresh medium to remove trace iron before production of conditioned medium. Serum free medium (DMEM and B27 without antioxidants) was applied to the C8B4 cells and the conditioned media were collected after 48 h. The media were cleared of debris by centrifugation and filtered before application to SH-SY5Y cells. The conditioned media were applied to the SH-SY5Y cells for 24 h after which time protein extracts were prepared from the cells. The level of expression of α-syn was determined by western blotting and detection with a specific antibody to α-syn. Conditioned medium from control microglia had no effect on α-syn expression while conditioned medium from iron-fed microglia showed an approximate three-fold increase in the level of α-syn detected (Figure 6A). In comparison, 50 μM ferric ammonium citrate had no effect on α-syn expression (Supplementary Figure S1). Additionally, conditioned medium from the microglia had no effect on SH-SY5Y cell viability (Supplementary Figure S2).

As the effect on α-syn expression may have been related to the use of microglial cell lines we sought to confirm the effect by using primary mouse microglia isolated from new-born mice. Mixed glial cultures were prepared and treated with 500 μM ferric ammonium citrate for at least two weeks. Microglia were then isolated and purified from these cultures (along with controls that were not iron-fed). Conditioned medium was then generated from these cultures and applied to SH-SY5Y for 24 h. Conditioned medium from iron-fed microglia but not controls caused an increase in expression of α-syn, as assessed by western blotting (Figure 6B).

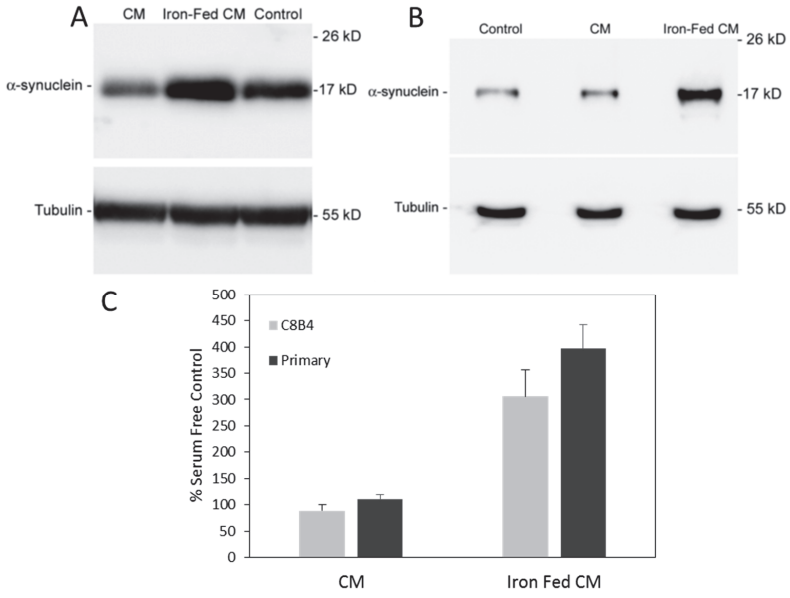

Figure 6. Increased levels of α-synuclein protein. SH-SY5Y (human neuroblastoma) cells were treated for 24 h with serum free medium (control) conditioned medium from control (CM) and iron-fed microglia (Iron-fed CM). The level of α-syn expressed by SH-SY5Y cells was assessed by western blot using a specific antibody. Protein loading levels were assessed using western blot for tubulin. The effect of conditioned medium from either (**A**) C8B4 or (**B**) Primary microglia was assessed and densitometric analysis carried out (**C**). Compared to SH-SY5Y cells grown in serum free medium (control) conditioned medium from control microglia (CM) had no significant effect ($p > 0.05$). In comparison, conditioned medium from iron-fed microglia (Iron-fed CM) caused a significant increase ($p < 0.05$) in the levels of α-syn protein detected. Conditioned medium from both C8B4 and primary microglia has a similar effect. Shown are the mean and S.E.M. of four separate experiments.

3.7. SNCA Promoter Activity Is Increased by Conditioned Medium from Iron-Fed Microglia

Change in α-syn expression may come from either increased transcription or decreased breakdown of the protein. The first step in assessing this process is to assay transcription of the protein from its gene. In order to do this, we used three luciferase reporter constructs based on the α-syn SNCA promoter [31]. The largest of these promoter fragments was 6.1 kb in size. Its 3′ end coincided with the start codon of the α-syn ORF and was termed −6.1/ATG (Figure 7). Two further constructs included smaller regions of this fragment. −6.1/−1.3 was missing a large 3′ section prior to the start codon and −4.1/ATG was missing 2 kb from the 5′ end. The fragments were cloned into a firefly luciferase expression vector and transiently transfected in SH-SY5Y cells in parallel with a renilla luciferase expressing plasmid driven by the thymidine kinase promoter (pTK) to control for differences in cell number and transfection efficiency. 24 h after transfection, the cells were treated with conditioned medium either from control or iron-fed C8B4 microglia plated at equivalent density. After 24 h a dual luciferase assay was performed on extracts from the SH-SY5Y cells. A significant increase in luciferase activity was seen with the reporters −6.1/ATG and −6.1/−1.3 when SH-SY5Y cells were treated with conditioned medium from iron-fed microglia (Figure 7). No change was observed when conditioned medium from control microglia was used. The smaller fragment −4.1/ATG showed a significant decrease in reporter activity when conditioned medium from both control and iron-fed microglia was applied. The response of this smaller fragment did not explain the differences in protein expression observed when conditioned medium from iron-fed microglia was applied to SH-SY5Y cells, whereas the response of the −6.1/−1.3 reporter fragment matched the increase. These results suggest that the increase in α-syn expression may be induced at the transcriptional level and it involve a transcription factor that binds between −6.1 and −4.1 in the SNCA promoter.

3.8. Neutralisation of Cytokines Released from Iron-Fed Microglia Blocks Increased α-Synuclein Expression

We measured changes in the level of cytokines that is released by iron-fed microglia, as indicated above. The most consistent change was in TNFα. There was also an increase in IL-1β for C8B4 microglia. Decreased cytokine levels were considered of no interest, as conditioned medium from control microglia had no effect on α-syn levels. Therefore, we attempted to neutralize the increase in α-syn that occurred in SH-SY5Y cells in response to treatment with conditioned medium from iron-fed microglia. Conditioned medium from iron-fed microglia was applied to SH-SY5Y cells as before. Some samples of conditioned medium were pre-treated with either an antibody to TNFα, IL-1β, or an anti-rabbit IgG (150 ng/mL). After 24 h, the level of α-syn protein in the cells was determined by western blot. Only neutralization with the TNFα antibody resulted in the reduction in measured α-syn levels (Figure 8). This implies that the increased level of α-syn induced by iron-fed conditioned medium is possibly caused by TNFα.

We tested whether TNFα or IL-1β could increase the expression of α-syn in SH-SY5Y cells. SH-SY5Y cells were treated with 50 nM TNFα, 50 nM IL-1β, or both in serum free medium supplemented with B27. After 24 h the levels of α-syn were assessed by western blot. Only TNFα caused a significant increase in α-syn levels (Figure 9A,B). The combination of TNFα and IL-1β was not significantly different to TNFα alone. We also tested the effects of the same cytokines on the activity of the SNCA promoter fragment −6.1/−1.3. The luciferase reporter fragment was transfected into SH-SY5Y cells and treated with 50 nM TNFα, 50 nM IL-1β, or both in serum free medium supplemented with B27. The measured luciferase activity showed a significant increase in the promoter activity in SH-SY5Y cells when treated with TNFα but not IL-1β (Figure 9C). These results combined suggest that the molecule released from iron-fed microglia that increased α-syn expression is TNFα.

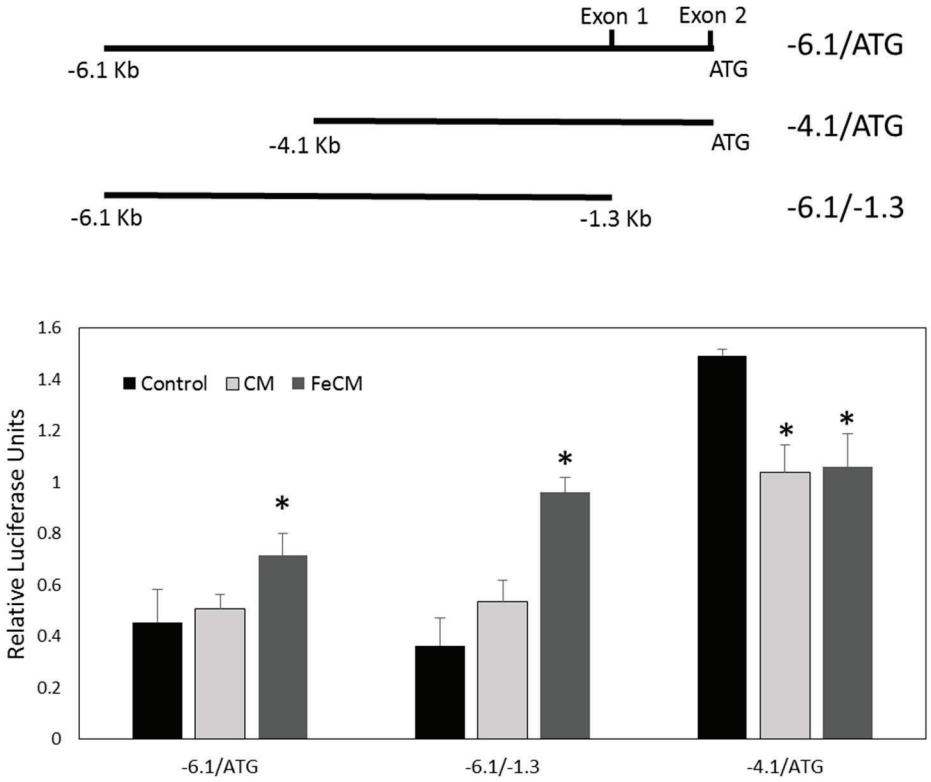

Figure 7. SNCA transcriptional activity. We wished to determine if the change in α-syn protein levels in cells treated with iron-fed microglial conditioned medium was due to a change in activity of the promoter of cytosolic protein alpha-synuclein (α-syn), SNCA. We used three reporter constructs containing fragments of the SNCA promoter that drive luciferase expression when transcriptionally active. The fragments are illustrated by the schematic which shows the overlap with the main features of the SNCA promoter. −6.1/ATG covers the majority of the promoter as well as the 5′ non-coding domain prior to the start codon. −6.1/−1.3 covers the same part of the promoter as −6.1/ATG but excludes the non-coding exons. The fragment −4.1/ATG excludes 2 kb of the sequence at the 5′ end of the promoter. SH-SY5Y cells were transiently transfected with the three constructs and the cells were treated for 24 h with either serum free medium (control), conditioned medium from C8B4 microglia (CM) or conditioned medium from iron-fed C8B4 microglia (FeCM). Readout of luciferase activity showed that conditioned medium from C8B4 microglia had no significant effect on the −6.1/ATG or −6.1/−1.3 promoter fragment, while iron-fed conditioned medium caused a significant increase ($p < 0.05$) in activity. In contrast the conditioned medium from both kinds of microglia decreased the activity seen with the −4.1/ATG fragment. The difference in activity suggests that the change in expression of α-syn may come from binding of a transcription factor between −6.1 and −4.1 on the SNCA promoter in response to a factor in iron-fed microglia conditioned medium. Shown are the mean and S.E.M. of four separate experiments.

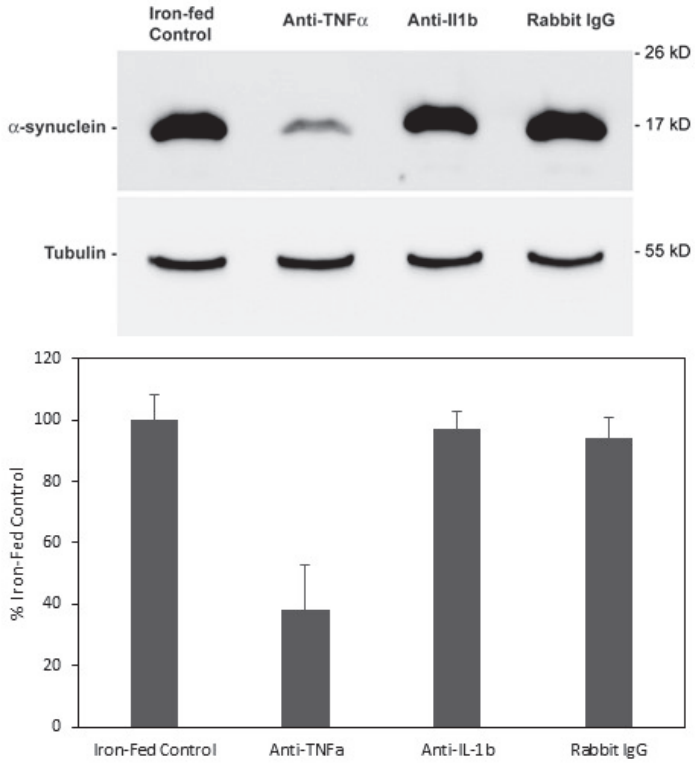

Figure 8. Cytokine Neutralization SH-SY5Y. Cells were treated with conditioned medium from iron-fed C8B4 microglia pre-treated with antibodies in an attempt to neutralize cytokines present that may be responsible for inducing increased expression of α-syn. We tested the effect of antibodies to TNFα, IL-1β and as a control, rabbit IgG. Medium was treated with 150 ng/mL of the specific antibody for one hour before applying to the SH-SY5Y cells. After 24 h western blot was used to assess the level of α-syn protein and tubulin to compare loading. On the antibodies tested only anti- TNFα caused a significant ($p < 0.05$) reduction in the level of α-syn detected. Shown are the mean and S.E.M. of four separate experiments.

3.9. Increased α-Synuclein Expression Induced by Iron-Fed Microglia Was Mediated by the NF-κB Pathway

The two predominant pathways that are involved in signalling to the nucleus via TNFα occur through either JNK(c-Jun N-terminal kinase)/AP-1 or NF-κB (nuclear factor kappa-B). Potentially, increased α-syn expression that is caused by the conditioned medium from iron-fed microglia could be mediated through either pathway (or both). We used inhibitors of both JNK and NF-κB to determine whether blocking these pathways could prevent the increase in α-syn that is caused by the conditioned medium. 200 nM JNK inhibitor II (CAS129-56-6) [34], 25 μM JNK inhibitor XV (IQ-1S, CAS1421610-21-0) [35], and 300 nM NF-κB inhibitor IV (CAS 139141-12-1) [36] were applied to SH-SY5Y cells treated with C8B4 iron-fed conditioned medium for 24 h. The concentrations used were based on concentrations used effectively in the cited papers. After western blotting and detection of α-syn, the strongest inhibitory effect on α-syn expression was seen with the NF-κB inhibitor IV (Figure 10). While significant inhibition was also seen with the JNK inhibitor XV, there was no significant inhibition with JNK inhibitor II. These results suggest that the increased expression of α-syn induced by the iron-fed microglia conditioned medium is mediated through NF-κB but with the possibility that some of the effect is also mediated by the JNK pathway.

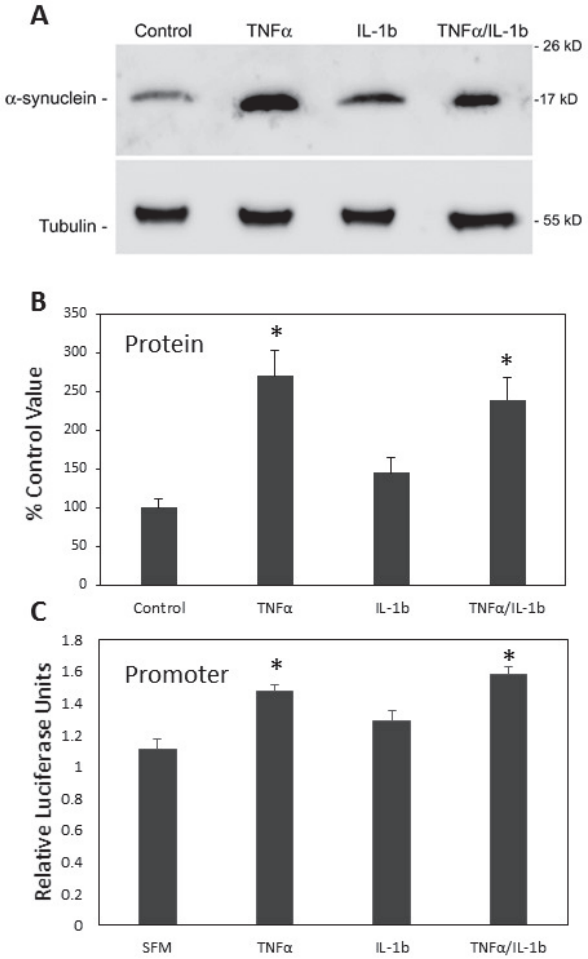

Figure 9. Cytokines and α-synuclein expression. (**A**) We tested cytokines to determine if they could induce increased expression of α-syn in SH-SY5Y cells. SH-SY5Y cells were grown in serum free medium and 50 ng/mL of either mouse TNFα, IL-1β, or both was applied to the cells twice in a 24 h period. Western blot was then carried out to assess α-syn expression levels. Tubulin was also assessed as a loading control. (**B**) Analysis showed that only TNFα or TNFα/IL-1β caused an increase in α-syn expression. As IL-1β had no significant effect this result was only due to the presence of TNFα. (**C**) We also tested the effects of the cytokines on the activity of the SNCA promoter reporter −6.1/−1.3 which showed the strongest response to iron-fed conditioned medium. The SH-SY5Y cells transiently transfected with the reporter were treated similarly with the cytokines. Luciferase activity was significantly ($p < 0.05$) increased only for cells treated with TNFα alone or in combination with IL-1β. Shown are the mean and S.E.M. of four separate experiments.

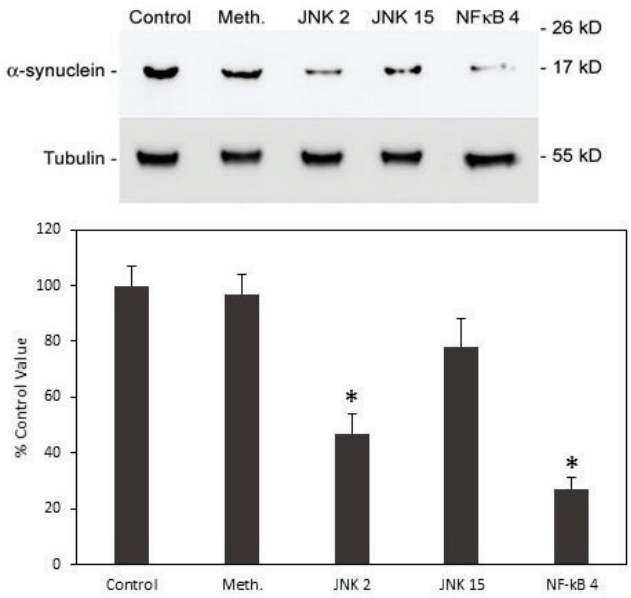

Figure 10. Inhibitors of signaling pathways. In order to assess the role of both the JNK pathway and NF-κB pathway in the increased expression of α-syn in response to conditioned medium from iron-fed C8B4 microglia, we applied a series of inhibitors of these pathways to SH-SY5Y cells during treatment with the conditioned medium. The compounds used were two inhibitors of the JNK pathways (JNK 2 and JNK 15) and one inhibitor of the NF-κB pathway (NF-κB 4). As the inhibitors were soluble in methanol we also included a methanol control at the highest volume used. After a 24 h treatment, the levels of α-syn and tubulin were assessed by western blot. The strongest inhibition was seen with the NF-κB 4 inhibitor. The inhibitory effect of JNK 2 was also significant ($p < 0.05$) but the effect of JNK 15 was not. Shown are the mean and S.E.M. of four separate experiments.

3.10. Conditioned Medium from Iron-Fed Microglia Caused a Decrease in α-Synuclein Activity

We have shown previously that α-syn is able to reduce iron through ferrireductase activity [37]. This activity is present in vivo [38] and the active form of the protein is a tetramer [29]. Tetrameric α-syn has also been suggested to be the native form of α-syn [39] and its loss from the cell might lead to the formation of disease specific oligomers [40]. We analysed ferrireductase activity in cells overexpressing α-syn when exposed to the conditioned medium from iron-fed microglia. Conditioned medium from C8B4 control microglia had no significant effect on the measured ferrireductase activity (Figure 11), whereas conditioned medium from iron-fed microglia caused a significant reduction in the activity measured. As ferrireductase activity is associated with the tetrameric form of the protein we also assessed whether there was a change in the level of tetramers present in α-syn overexpressing cells that are exposed to conditioned medium from iron-fed microglia. α-syn tetramers can be identified in SH-SY5Y cells by cross-linking protein extracts from the cells. Tetramers can then be observed by western blot. SH-SY5Ys that are treated with conditioned medium from iron-fed microglia showed significantly reduced levels of tetramers when compared to cells treated with conditioned medium from control microglia (Figure 11). This data suggests that conditioned medium from iron-fed microglia reduced the formation of α-syn tetramers, and consequentially reduced the ferrireductase activity of α-syn measured in SH-SY5Y cells.

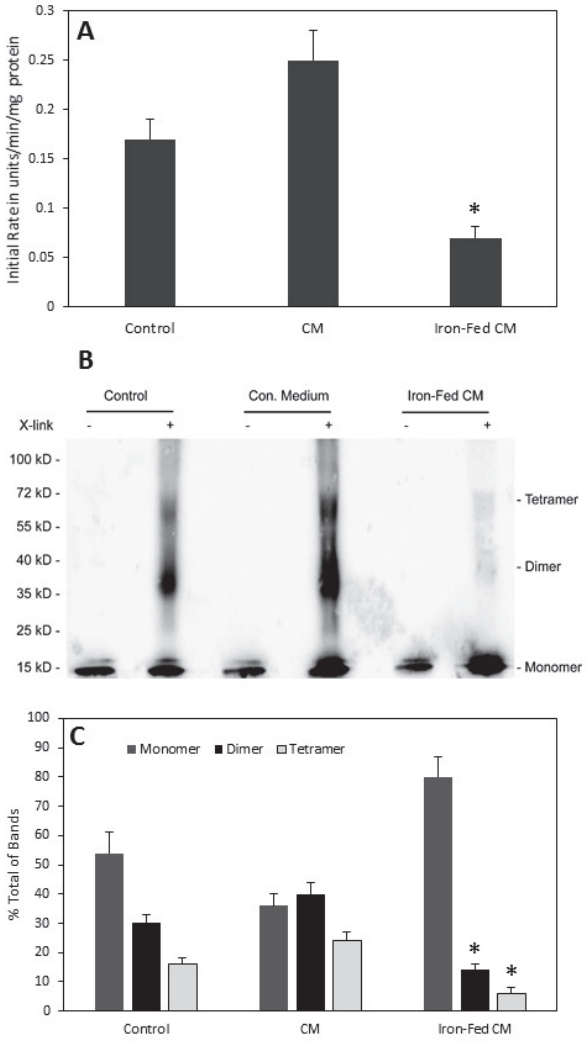

Figure 11. Ferrireductase activity and tetramers. (**A**) α-syn possesses ferrireductase activity and SH-SY5Y cells overexpressing α-syn show increased levels of iron reduction. We measured the ferrireductase activity in SH-SY5Y cells stably overexpressing α-syn. The cells were exposed to conditioned medium from either control C8B4 microglia or iron-fed microglia for 24 h. Protein extracts were made and the initial rate determined for a concentration of 500 μM ferric ammonium citrate using a standard ferrireductase assay based on colour change associated with the binding of Fe(II) to ferrozine. The results show that conditioned medium from iron-fed microglia significantly ($p < 0.05$) decreased measured ferrireductase activity while that from control microglia did not. Shown are the mean and s.e. of four separate experiments. (**B**,**C**) α-syn ferrireductase activity has been shown to be due to the presence of tetramers of α-syn in the cell membrane. We therefore measured the presence of α-syn tetramers in membrane extracts of α-syn overexpressing SH-SY5Y cells. Protein extracts were prepared from membrane fractions and cross-linked with DSS. Western blot was then used to assess the presence of monomers, dimers and tetramers of α-syn in the extracts. While treatment with C8B4 conditioned medium had no effect on the ratio of tetramers to dimers and monomers, treatment with conditioned medium from iron-fed C8B4 microglia caused a significant ($p < 0.05$) reduction in the levels of both tetramers and dimers. Shown are the mean and S.E.M. of four separate experiments.

3.11. Aggregation of α-Synuclein

The aggregation of α-syn into oligomeric species is considered to be a major hallmark of diseases that are associated with α-syn. Aggregates of α-syn can frequently be detected in cells overexpressing the protein. We had previously developed a western blot assay to detect oligomeric aggregates

of α-syn [26]. SH-SY5Y cells overexpressing α-syn were treated with conditioned medium from primary control microglia or iron-fed microglia for 24 h. Extracts were prepared from the SH-SY5Y cells and electrophoresed on a PAGE gel. After western blot and detection with a specific antibody, high molecular weight (>300 kD) bands of α-syn oligomers were detected. Treatment with control microglia conditioned medium had no significant effect on the levels of aggregates detected (despite a high level of variability). However, treatment with conditioned medium from iron-fed microglia results in a large and significant increase in the level of aggregates detected (Figure 12). This implies that conditioned medium from iron-fed microglia is able to induce the aggregation of α-syn independently of its effect on protein expression. A similar result was observed with conditioned medium from C8B4 cells (data not shown).

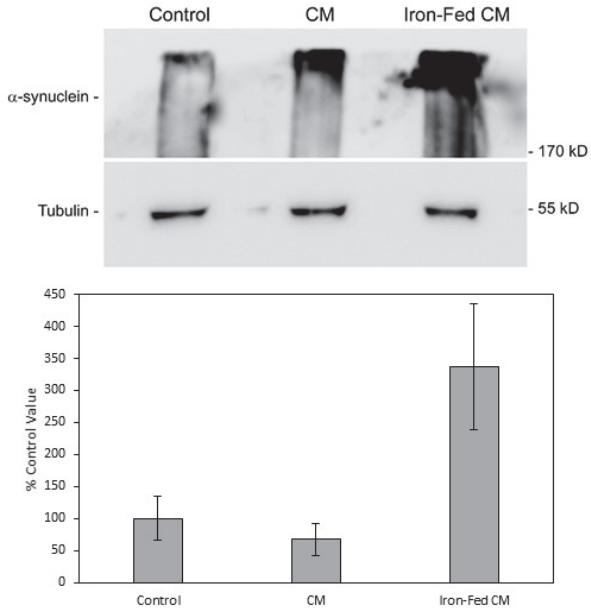

Figure 12. Aggregation of α-syn. Western blot was used to detect high molecular weight aggregates of α-syn in extracts from SH-SY5Y cells treated with conditioned medium from primary microglia. SH-SY5Y cells were treated for 24 h with medium from control microglia (CM) or iron-fed microglia (Iron-Fed CM). Some cells were treated with just serum free medium (control). Extracts were prepared and electrophoresed on a 6% PAGE gel. The protein was then transferred by blot to a PVDF membrane (3 h, 100 mA) and α-syn detected with a specific antibody. High molecular weight bands for α-syn were indicative of aggregates. We also verified protein loading by re-probing the same blots for tubulin. Bands for α-syn were then analysed densitometrically. Values for control were normalised to 100% and values for the treated samples compared. Only treatment with iron-fed conditioned medium caused a significant ($p < 0.05$) increase in α-syn detected in the aggregate band. Shown are the mean and S.E.M. for four separate experiments.

4. Discussion

The aim of the work presented here was to establish a model of microglia that could be used to replicate aspects of the aging brain. The study of both normal aging and neurodegenerative diseases is compromised by the lack of effective models of the aging brain. Neurodegenerative diseases are predominantly associated with aging, as it is considered the single most important risk factor for their development. However, studies rarely ever incorporate conditions related to the aging phenotype. This is particularly hampered by poor definition of exactly what changes are important to consider. Numerous reports described changes in oxidative stress [41], by-products of oxidative damage [42], or trace metals that can themselves induce oxidative stress and subsequent

by-products [43]. Here, we have utilized knowledge of the phenotype of dystrophic microglia to modify a microglial cell line and combine it with the study of disease-associated changes in α-syn.

Developing a model of senescent/dystrophic microglia in vitro has numerous issues. Chief among these is the lack of clarity in defining dystrophic microglia [13]. There is currently no single molecular marker that would define a dystrophic or senescent microglial cell. Proteomics/transcriptomics based studies comparing microglia from old and young brains have been carried out for both mouse and human but have yielded conflicting results [44–47]. However, there is a general cellular senescence signature that all cells show and this is no different for microglia, which also show characteristics aligning with a senescence-associated secretory phenotype [48].

The second hurdle for generating an in vitro model is the difficulty in using primary microglia. Isolated primary microglia rapidly change phenotype [49–51], microglia from adult mice are difficult to maintain [52] (there are still very few studies using them), and pushing microglia into a senescent/dystrophic phenotype reduces both their yield and viability. For these reasons, we predominantly used a microglial cell line as this allowed for us to generate large numbers of microglia with our iron-fed phenotype.

Dystrophic microglia in the aging brain do have a number of clear differences to normal resting microglia. These include morphological changes, such as loss of processes, increased iron storage, and increased expression of ferritin [9,10,13,53–55]. Of considerable interest to us was the evidence for increased iron storage. While in a normal aging situation the accumulation of iron is likely a consequence of the aging process, iron is highly associated with the risk of oxidative damage, a hallmark of aging. Therefore, a possibility exists that increasing iron storage experimentally would induce the changes observed in dystrophic microglia in vivo. There is already considerable evidence that iron-overload can induce senescent changes in cells including microglia [56–58]. In this light, our finding that cultured microglia maintained in a high iron environment adopt characteristics of dystrophic microglia is not surprising.

As our iron-fed microglia are a model of dystrophic microglia they also demonstrate characteristics of the senescence-associated secretory phenotype (SASP) referred to previously. This includes the reduced proliferation and increased release of pro-inflammatory cytokines such as TNFα. A recent study has suggested that the potassium channel Kv1.3 is increased in expression in aged microglia [33]. This was a finding we also confirmed in our iron-fed microglia. Further study from the same group showed that in aged mice, Kv1.3 was associated with release of pro-inflammatory cytokines including TNFα [59]. Therefore, increased expression of Kv1.3 and its subsequent down-stream effects are likely a part of SASP.

As mentioned above, our aim was to develop a model of aged microglia that could be applied to the study of neurodegenerative diseases. Microglia and also dystrophic microglia have been implicated in many neurodegenerative diseases [5,55,60]. We chose to concentrate on synucleinopathies on the basis of our previous experience studying the role of α-syn in disease models. There is strong evidence that microglia play a role in synucleinopathies, such as PD [61], multiple system atrophy [62], and Dementia with Lewy Bodies [24,63]. There is currently also considerable interest in the impact of age-related changes to microglia in PD [8,64] and it has even been suggested to be causative of neurodegeneration in the *substantia nigra* [23,65].

Synucleinopathies are associated with the aggregation of α-syn in cells and this is believed to stem from two causative processes. The first and most well recognized is an increased expression of α-syn, resulting in molecular crowding [66,67]. The second and more controversial is the more recent suggestion that the native and functional form of α-syn is a tetramer and loss of tetramer formation increases the likelihood of aggregation [40]. Using conditioned medium from our model dystrophic microglia, we were able to induce increased α-syn expression, reduced tetramer formation and increased aggregation in SH-SY5Y cells. Thus, by the incorporation of an aspect of brain aging we were able to induce several aspects of the disease state in neuronal cells. For this reason, we believe

that we have developed a simple and valuable tool for the exploration of the molecular mechanisms behind synuclein related diseases and possibly other neurodegenerative diseases.

The ability of conditioned medium from iron-fed microglia to induce changes in α-syn in SH-SY5Y cells implies that a soluble factor released by the microglia is responsible. We showed that the levels of iron released from the iron-fed microglia are small and application of iron to SH-SY5Y does not cause the same response. There is also no evidence in the literature that iron levels alter α-syn transcription. In contrast, we were able to show that iron-fed microglia release increased levels of TNFα, neutralization of TNFα blocks the increased α-syn expression and that exogenous TNFα also induced increased α-syn expression and transcription. This implies that TNFα mediates the effects we observed. This result is further supported by findings showing that an inhibitor of NF-κB blocks the increased expression as well. TNFα effects on protein expression via transcriptional activation are frequently mediated through an NF-κB controlled pathway [68]. There have been few papers linking TNFα and α-syn, but one paper has shown that TNFα increases α-syn in control human iPS cells [69]. Another paper did suggest that TNFα released by microglia could impair autophagic flux and this increased α-syn levels through decreased breakdown of the protein [70]. It is also possible that decreased autophagy could contribute to the increased levels of α-syn that we observed. Similarly, there been very few reports that α-syn expression can be increased by NF-κB, even though it has been reported there are NF-κB binding sites on SNCA [69]. Our data showed that there is a fragment of the SNCA promoter that appears to be necessary for TNFα-driven induction of expression (between −6.1 and −4.1 kb) and analysis with online transcription factor binding site software (Match, Biobase, Germany) identified at least three potential binding sites in this region for NF-κB. Analysis of patients with PD has shown increased TNFα levels and increased nuclear localisation of NF-κB in neurons and microglia in the substantia nigra [71]. In general, there is a significant amount of data implying that NF-κB activation might be relevant to PD [72–74]. This suggests our model might have relevance to the changes observed in PD.

TNFα is not the only cytokine that is altered in Parkinson's disease [75]. Many studies look at cytokines that are released by microglia in response to α-syn rather than changes in microglial released cytokines that could alter neuronal activity in PD [76]. In this regard, aggregates of α-syn induced neurotoxic effects that are mediated by microglia through the activation of Toll-like receptor 2 [77]. We also observed other changes in cytokines other than TNFα. However, these appeared to have no impact on α-syn expression such as IL-1β or were higher in control microglia than the iron-fed ones. In this case, as we saw no change in α-syn expression when treated with control conditioned medium versus untreated, the levels of these cytokines, regardless of how they changed, had no impact on α-syn. While the microglia that we used were murine in origin and the SH-SY5Y cells were human, most murine cytokines are able to bind to human cytokine receptors, the most notable exception being IL-10 where the murine form cannot bind to the human IL-10 receptor [78].

We noted other changes in α-syn that we have not linked to TNFα. These include tetramer formation and ferrireductase activity. However, the regulation of both of these aspects of α-syn activity is currently unknown. We have previously shown that tetramer formation is connected to increased ferrireductase activity, while increased aggregation is linked to a loss of this activity [29,38]. The process of aggregation of α-syn is likely to require the protein to initially pass through small oligomeric states. However, the stable tetramer that is expressed in cells is supposed to be highly structured with a helical configuration and its formation is suggested to prevent higher order oligomerization and aggregation [39,79]. We also observed that conditioned medium from iron-fed microglia induced increased levels of aggregation. Aggregation of α-syn into potentially toxic oligomeric species is considered to be one of the hallmarks of synucleinopathies [80,81]. As this is accompanied by a reduction in tetramer formation, this change possibly represents the mechanism by which aggregation could occur in diseases, like PD. This model system therefore may be of benefit in analysing the mechanics of conversion of α-syn from its normal cellular isoform to the oligomeric aggregates generated in disease.

5. Conclusions

In summary, we have created a model to incorporate an aspect of brain aging into the study of α-syn. This model is able to recapitulate a number of changes that are observed in diseases, like Parkinson's disease. These changes include increased expression, aggregation, reduced tetramer formation and ferrireductase activity of α-syn. The model incorporates a potential in vitro dystrophic microglia component. By overloading microglia with iron, we have shown that they behave similarly to dystrophic microglia, altering their morphology, iron storage, protein expression and cytokine release. The utility of these model dystrophic/senescent microglia will allow for further study of both the ontology of dystrophic microglia and the potential role of such microglia in neurodegenerative disease. This may allow for more robust in vitro models for the study of these complex diseases.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/8/3/67/s1. Supplementary Figure S1 Iron and α-synuclein expression, Supplementary Figure S2 Toxicity of Conditioned Medium.

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Supplementary Data

Model Senescent Microglia Induce Disease Related Changes in Alpha-synuclein Expression and Activity.

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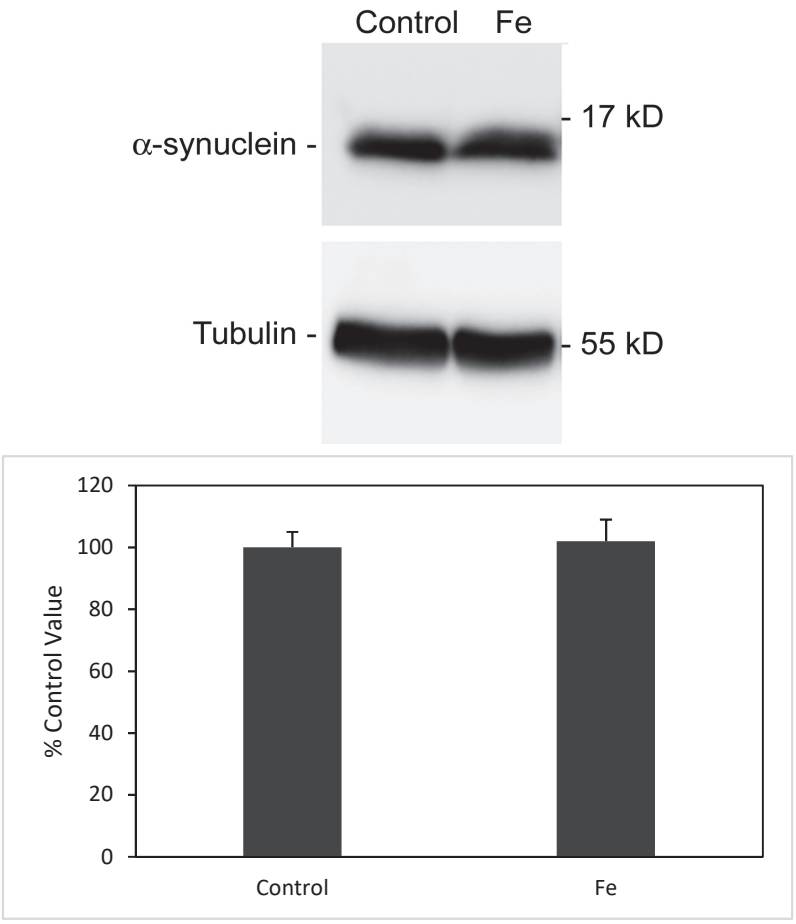
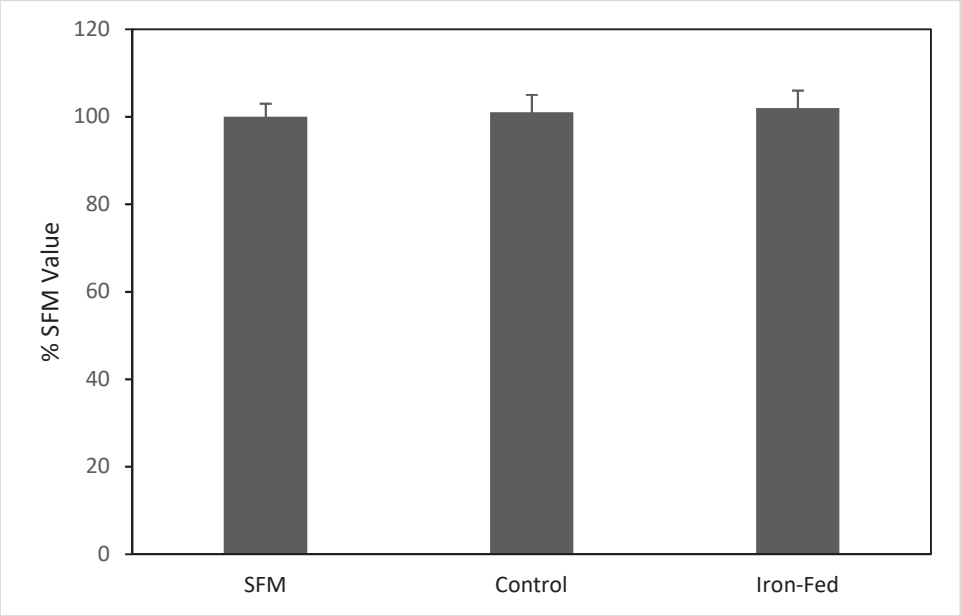


Supplementary Figure 1 Iron and α-synuclein expression.

SH-SY5Y cells were grown in serum free medium for 24 h. Control cells were grown only in the serum free medium while iron treated cells (Fe) were grown in 50 µM ferric ammonium citrate. After 24 h the cells were harvested, and protein extracted. Equal amounts of protein were eletrophoresed on a 14% PAGEgel. After semi-dry transfer to a membrane α-syn was detected with a specific antibody (MJFR1) and bands detected with chemiluminescence. The detected bands were quantitated densitometrically. The process was repeated after stripping the blot and tubulin was detected with a monoclonal antibody. Treatment with iron had no effect on the levels α-syn detected. Shown are the mean and S.E.M for four experiments.



Supplementary Figure 2: Toxicity of conditioned medium.

The toxicity of conditioned medium from C8B4 microglia was tested on SH-SY5Y cells. The cells were treated for 24 h with either serum free medium (SFM), conditioned medium from control microglia (Control) or iron-fed microglia (Iron-Fed). The survival after 24 h was determined using an MTT viability assay. Neither the medium from control microglia nor iron-fed microglia had any significant ($p > 0.05$) effect on SH-SY5Y cell viability when compared to SFM. Shown are the mean and S.E.M for four separate experiments with three replicates each.

3.5 Concluding commentary

The findings of the previous chapter suggest that the changes in microglia induced by iron are broadly replicated between species and in primary culture. This suggests that our model of senescent microglia is robust and reproducible can be used in different model systems with similar effect.

Although the changes we saw in human and mouse microglial cells were similar, some discrepancies were noted. Namely, differences in cytokine production were present in human and mouse microglia. Even though the cytokine expression profile in both iron-fed human and mouse microglia was decidedly pro-inflammatory, no changes in TNFα levels in iron-fed human microglia were reported while in Chapter 3 elevated TNFα was central to the effect observed on α-synuclein. As TNFα secretion is known to be increased in aged human microglia and in the context of PD it is possible that the very low TNFα levels seen in the human microglial cell line are a feature of this specific cell line.

Figure 3.1 shows an alternative western blot to Figure 10 presented in the paper and illustrates that the NF-κB and possibly the JNK pathways are likely involved in mediating the effect of TNFα on α-syn.

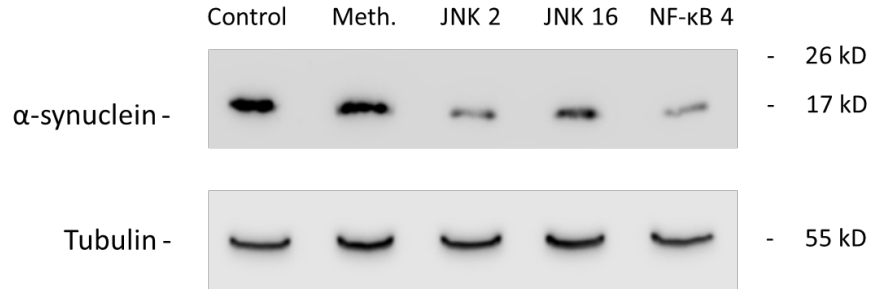


Figure 3.1 Inhibitors of signalling pathways. In order to assess the role of both the JNK pathway and NF-κB in the increased expression of α-syn in response to conditioned medium from iron-fed microglia, we applied a series of inhibitors of these pathways to SH-SY5Y cells during treatment with the conditioned medium. The compounds used were two inhibitors of the JNK pathways (JNK 2 and JNK 15) and one inhibitor of the NF-κB pathway (NF-κB 4). As the inhibitors were soluble in methanol, we also included a methanol control at the highest volume used. After a 24 h treatment the levels of α-syn and tubulin were assessed by western blot.

The effects of microglial TNFα on α-synuclein in neuronal cells shown in this paper provide a link between age-related changes in microglia and the pathology of PD as it demonstrates that a cytokine released by aged microglia has the ability to increase the expression of α-synuclein. The downstream effects of elevated α-synuclein levels will be investigated in the work presented in the next chapter.

4. Levels of α- and ß-synuclein regulate cellular susceptibility to toxicity from α-synuclein oligomers

4.1 Introductory commentary

In chapter 3 it was demonstrated that the senescent microglia model can increase α-synuclein levels in SHSY5Y neuronal cells. Increased α-synuclein expression has been linked to the pathology of PD and other synucleinopathies.

In this paper we investigate how increased expression of α-synuclein can affect neurons and how those effects are mediated. It was found that elevated levels of α-synuclein affect the susceptibility of SHSY5Ys to toxic α-synuclein oligomeric species generated *in vitro* using an MTT assay. This was investigated not only in cells that overexpressed α-synuclein but also in cell lines overexpressing structural mutant forms of α-synuclein to understand whether particular domains of α-synuclein play a role in the susceptibility to toxic oligomers. β-synuclein has been reported to be protective against α-synuclein neurotoxicity. β- and γ-synuclein were also overexpressed and their effects on neuronal viability were compared.

As detailed in Chapter 1 FOXO3a is a transcription factor whose activity is heavily implicated in neurodegeneration including PD and in aging. FOXO3a can trigger neuronal apoptosis. Therefore it was highly likely that FOXO3a activity mediated the neuronal toxicity caused by elevated α-synuclein levels. We investigated the FOXO3a pathway in the context of α-synuclein toxicity by measuring FOXO3a levels with western blotting and inhibiting FOXO3a. Inhibition of FOXO3a in SHSY5Y cells was protective against α-synuclein toxic oligomers. As presented in chapter3 increased levels of α-synuclein resulted in elevated ferrireductase activity. In this chapter we investigated whether the increased levels of Fe^{2+} generated by α-synuclein can increase FOXO3a activation by altering iron levels in the cell and measuring FOXO3a levels by western blot. We found that both increased iron and overexpressing the ferrireductase Steap3 resulted in elevated FOXO3a levels while elevated β-synuclein did not.

4.2 Statement of contribution

This declaration concerns the article entitled:									
Levels of α- and β-synuclein regulate cellular susceptibility to toxicity from α-synuclein oligomers									
Publication status (tick one)									
draft manuscript		**Submitted**		**In review**		**Accepted**		**Published**	X
Publication details (reference)	Angelova, D, Jones, H & Brown, D 2018, 'Levels of alpha- and beta-synuclein regulate cellular susceptibility to toxicity from alpha-synuclein oligomers.' FASEB Journal, vol. 32, no. 2, pp. 995-1006. DOI: 10.1096/fj.201700675R								
Candidate's contribution to the paper (detailed, and also given as a percentage).	The candidate contributed to/ considerably contributed to/predominantly executed the… Formulation of ideas: Taking the research further with ideas for the pathway involved in the phenomenon observed. (50%) Design of methodology: Modification of cell culture, western blot and kit assay protocols to obtain quality data. (50%) Experimental work: Generated data in figures 3,5,6 and 8. (50%) Presentation of data in journal format: Created some figures and proof-read the manuscript. (50%)								
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.								
Signed	Dafina Angelova					**Date**	18.09.18		

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4.4 Levels of α- and ß-synuclein regulate cellular susceptibility to toxicity from α-synuclein oligomers

Dafina M. Angelova, Hannah B.L. Jones and David R. Brown

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Levels of α- and β-synuclein regulate cellular susceptibility to toxicity from α-synuclein oligomers

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ABSTRACT: α-Synuclein (α-syn) is associated with a range of diseases, including Parkinson disease. In disease, α-syn is known to aggregate and has the potential to be neurotoxic. The association between copper and α-syn results in the formation of stellate toxic oligomers that are highly toxic to cultured neurons. We further investigated the mechanism of toxicity of α-syn oligomers. Cells that overexpress α-syn showed increased susceptibility to the toxicity of the oligomers, while those that overexpressed β-syn showed increased resistance to the toxic oligomers. Elevated α-syn expression caused an increase in expression of the transcription factor Forkhead box O3a (FoxO3a). Inhibition of FoxO3a activity by the overexpression of DNA binding domain of FoxO3a resulted in significant protection from α-syn oligomer toxicity. Increased FoxO3a expression in cells was shown to be caused by increased ferrireductase activity and Fe(II) levels. These results suggest that α-syn increases FoxO3a expression as a result of its intrinsic ferrireductase activity. The results also suggest that FoxO3a plays a pivotal role in the toxicity of both Fe(II) and toxic α-syn species to neuronal cells.—Angelova, D. M., Jones, H. B. L., Brown, D. R. Levels of α- and β-synuclein regulate cellular susceptibility to toxicity from α-synuclein oligomers. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: FoxO3a · iron · Parkinson disease

α-Synuclein (α-syn) is associated with a number of neurodegenerative diseases, including Parkinson disease (PD), dementia with Lewy bodies, the Lewy body variant of Alzheimer disease (AD), and multiple system atrophy. Fibrillar aggregates of α-syn are the main constituent of Lewy bodies and Lewy neurites associated with these diseases (1, 2). Extracellular α-syn is present as aggregates in both the *substantia nigra* of patients with PD (2) and senile plaques of AD brains in the form of the non-Aβ component of AD, known as NAC (3, 4). Clear links between α-syn and neurodegeneration have been found. Neuronal cell loss and Lewy body–like inclusions occur in animal models overexpressing α-syn (5), and the rescue of dopaminergic cells from death occurs after down-regulation of α-syn expression in the *substantia nigra* of a PD rat model (6). Inherited mutations in familial cases of PD also illustrate the importance of α-syn to pathology. Inherited cases are linked to both point mutations (7, 8), leading to single point changes in the protein sequence (*e.g.*, A30P, E46K, A53T), and triplication of the α-syn gene (*SNCA*) locus (9).

The prevalence of fibrillar aggregates of α-syn associated with neurodegenerative diseases has led many authors to hypothesize that the aggregates cause cell death (1, 10, 11). However, the survival of neurons with intracellular Lewy bodies shows that the presence of intracytoplasmic α-syn aggregates is not toxic to all cells (2). Considerable evidence suggests that oligomers, formed as prefibrillar intermediates, may be the toxic component (12–14). In addition, there is evidence that extracellular α-syn is neurotoxic. Recombinant α-syn, which readily assembles into filaments *in vitro* with similar morphology, staining, and structure to α-syn filaments extracted from diseased brains, is toxic to cells when added to the culture medium, particularly in its aggregated form (15–22). The higher levels of α-syn oligomers in cerebrospinal fluid of patients with PD support a hypothesis that extracellular α-syn oligomers may be neurotoxic (23). However, the toxic species and mechanism of toxicity are still unclear.

Although there is considerable and extensive evidence for the role of aggregates of α-syn in a variety of diseases, there is less evidence for its normal cellular role. There are currently two theories about the function of α-syn. The first suggests that it mediates the release of dopamine (24), while the second and more recent suggests that it enzymatically reduces iron (25). There is considerable evidence

ABBREVIATIONS: AD, Alzheimer disease; α-syn-TO, toxic oligomers of α-syn; Δ2–9, mutant of α-syn or β-syn lacking amino acid residues 2–9; Δ2–9/H50A, mutant of α-syn lacking amino acid residues 2–9 and with histidine residue at position 50 mutated to alanine; Δ2–9/H65A, mutant of β-syn lacking amino acid residues 2–9 and with histidine residue at position 65 mutated to alanine; DBD, DNA-binding domain; FoxO3a, Forkhead box O3a; H50A, histidine residue at position 50 mutated to alanine in α-syn; H65A, histidine residue at position 65 mutated to alanine in β-syn; MTT, (4,5-dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide; ORF, open reading frame; PD, Parkinson disease; p-FoxO3a, phosphorylated FoxO3a; ROS, reactive oxygen species; syn, synuclein; T-FoxO3a, all forms of FoxO3a; ThT, thioflavine T; WT, wild type

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that α-syn binds to copper and iron (25–30). α-Syn has been shown to be a ferrireductase both *in vivo* and *in vitro*, and it has also been shown to have reduced activity in PD (31). Detail kinetics studies have shown α-syn ferrireductase activity is regulated by substrate inhibition and is membrane associated, and that the active form is a tetramer (32). Overall, there is strong evidence for a link between α-syn and iron metabolism.

There is compelling evidence for a role in many neurodegenerative diseases for the loss of homeostasis of the redox active transition metals iron and copper and the resulting oxidative stress. High levels of copper, zinc, and iron are found in and around amyloid plaques in AD brains (33). In PD brains, high levels of iron and zinc are found in the *substantia nigra* (34) and high levels of copper in the cerebrospinal fluid (35). While α-syn binds to copper and iron (25–30), α-syn aggregation is also stimulated in the presence of these metals (26, 29, 36). This has led us to examine whether the toxicity of extracellular synuclein proteins is exacerbated in the presence of metals. We have shown that the toxicity of α-syn aggregates increases in the presence of metals, in particular copper (37). This effect was not replicated with the α-syn homologs β-syn or γ-syn. The toxicity is caused by unique stellate soluble α-syn oligomers formed through morphologic change in the presence of copper. Our findings suggested that oligomerization of α-syn, combined with a loss of metal homeostasis, may be a key to the neurodegeneration observed in these diseases.

While initiation of cell death is a critical point in understanding the possible role of α-syn in cell loss in diseases like PD, cell death execution is possibly more interesting for potential intervention. From this point of view, the Forkhead box transcription factor family has gained increasing prominence both in the study of aging and neurodegeneration (38, 39). The relevance of Forkhead box O3a (FoxO3a) to the study of α-syn and PD has recently been shown by two articles. The first demonstrated increased and ectopic expression of FoxO3a in PD brains (40). The second study, using transgenic rats, demonstrated that the level of α-syn–induced neuron loss in the *substantia nigra* was increased by increase FoxO3a expression or reduced by overexpression of the DNA binding domain of FoxO3a (41). Therefore, understanding the potential role of FoxO3a in α-syn toxicity is of great importance.

In the current study, we found that FoxO3a plays a major role in toxicity induced by aggregated α-syn. Levels of expression of FoxO3a were modulated by levels of iron, which were dependent on ferrireductase activity of cellular α-syn. We found that the relative expression levels of both α-syn and β-syn play a critical role in susceptibility of SH-SY5Y cells to the toxicity of exogenous α-syn oligomers. These findings may have important implications for neuronal loss in PD and other neurodegenerative diseases.

MATERIALS AND METHODS

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Purification of synuclein proteins

Expression and purification of recombinant α-syn and its mutations were as previously described (30). Using pET expression vectors in BL21 *Escherichia coli* cells, untagged synuclein protein expression was induced at OD_{600} 0.5 to 1.0 with 1 mM isopropyl β-D-1-thiogalactopyranoside for 4 h. Cells were collected by centrifugation (8000 *g*) and lysed mechanically in 20 mM Tris-HCl/1 mM EDTA/pH 8.0 (buffer A), 1 mM PMSF, and 50 μg/ml DNase. Streptomycin sulfate was added to a final concentration of 1% to the lysate solution, then centrifuged at 8000 *g*. Ammonium sulfate (0.295 g/ml) was added to the supernatant (15% w/v solution) and stirred at 4°C for at least 1 h. After centrifugation at 10,000 *g*, the pellet was resuspended 50 ml buffer A. The semipurified lysate solution was loaded onto a 50 ml Q Sepharose column (Amersham Biosciences, Little Chalfont, United Kingdom). The column was washed with 100 ml buffer A; then a 2-column volume isocratic elution step to 25% buffer A + 1M NaCl (buffer B) was performed. Synuclein proteins were eluted with a broad-gradient elution (10 column volumes) from 25% buffer B to 50% buffer B (all synuclein proteins eluting at ~350 mM NaCl). SDS-PAGE analysis of Q Sepharose fractions was performed, and fractions enriched for synuclein were pooled. Synuclein proteins were collected as flowthrough from a PM30 cellulose membrane (EMD Millipore, Billerica, MA, USA), then concentrated with a PM10 PES membrane (EMD Millipore). Purified synuclein proteins were dialyzed extensively at 4°C in Chelex-treated Milli-Q (EMD Millipore). Protein concentration was measured by absorbance at 275 nm with extinction coefficient 5600 M/cm for α-syn (and mutants) and β-syn.

Production of synuclein fibrils

Five hundred microliters of 20 μM synuclein proteins in 10 mM Tris pH7.4 was formed into aliquots into 1.5 ml screw-capped tubes. $CuCl_2$ was added to a final concentration of 100 μM. After incubation at room temperature for 45 min, tubes were laid flat on an orbital shaker and incubated at 37°C shaking at 600 rpm for 6 d. Formation of fibrils was monitored using thioflavine T (ThT). Five microliters of each sample was mixed with 95 μl 10 μM ThT in 10 mM Tris pH7.4. Increased ThT fluorescence, indicating increased β-sheet structures, was monitored in 96-well plates with the FluoStar Omega (BMG Labtech, Ortenberg, Germany).

Cell culture

SH-SY5Y (human neuroblastoma) cells were cultured in 45% DMEM/45% Ham F-12 (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum and penicillin–streptomycin. Cells were maintained at $1 \times 10^6/75$ cm^2 at 37°C and 5% CO_2 in a humidified incubator. The neuronal status of SH-SY5Y cells was monitored by reverse transcription PCR with primers for tyrosine hydroxylase, dopamine transporter, and vesicle monamine transporter 2.

Cell lines derived from SH-SY5Y cells and overexpressing α-syn, β-syn, or mutations of either were developed by stable transfection of plasmids (pCDNA3.1) containing the open reading frame (ORF) of either protein using Fugene (Promega, Madison, WI, USA). The cell lines generated were as previously described (42).

(4,5-Dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide assay

For toxicity experiments, cells were plated at 2×10^5 cells per well of a 24-well plate in DMEM (Lonza) supplemented with 10% fetal

bovine serum and penicillin–streptomycin, then grown overnight. Cells were treated for 48 h with recombinant proteins at different concentrations. The (4,5-dimethylthiazol-2-*yl*)-2,5-diphenyltetrazolium bromide (MTT) reagent was resuspended at 2.5 mg/ml in water. Medium and treatments were removed from the wells, and 0.5 ml 50 μl of MTT in Hanks solution was added per well and incubated for 30 min. After removal of the MTT solution, cells and the resulting reduced tetrazolium were solubilized in 800 μl DMSO per well. Readings were taken at 570 nm using FLUOstar Omega (BMG Labtech). Each treatment was conducted in triplicate, averaged, and represented as the percentage of untreated control (vehicle alone). Each experiment was repeated 3 to 5 times.

Western blot analysis

Cells were lysed in 0.5% Igepal CA-630 and Complete protease inhibitor cocktail (Roche, Basel, Switzerland), sonicated 3 × 3 s on ice, and centrifuged 10,000 *g* for 3 min to remove insoluble membranes. Protein concentration was determined with a Bradford protein assay (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Protein concentrations were normalized and boiled for 5 min with 1× Laemmli SDS-PAGE buffer. Samples were loaded into either a 10% (for FoxO3a) or a 12% (for α-syn) acrylamide SDS-PAGE gel, with a buffer of Tris (250 mM) + glycine (1.92 M) + SDS (0.1% w/v), run at 250 V and 35 A for 45 min. Separated proteins were transferred to a PVDF membrane by a semidry transfer apparatus, then run at 25 V and 100 A for 1.5 h. Membranes were blocked in 5% w/v nonfat milk powder in Tris-buffered saline and Tween 20 for 30 min, incubated with primary antibody for 1 to 2 h, and washed 3 × 5 min in Tris-buffered saline and Tween 20. Membranes were blocked again and incubated with horseradish peroxidase–conjugated secondary antibody for 1 h. A further 3 × 10-min washes were performed, and the membranes were developed with Luminata Crescendo or Luminata Forte ECL substrate (Thermo Fisher Scientific, Waltham, MA, USA), then imaged with a Fusion SL CCD imaging system (Vilber Lourmat, Collégien, France).

Rabbit monoclonal anti–α-syn (MJFR1, immunogen human α-syn 1–150; Abcam, Cambridge, United Kingdom) was used for human α-syn detection at a dilution of 1:4000. Total FoxO3a (75D8) and phosphorylated FoxO3a (p-FoxO3a; ser253) were detected with rabbit antibodies at 1:1000 (Cell Signaling Technology, Danvers, MA, USA). Mouse monoclonal anti–α-tubulin (T5186; Sigma-Aldrich, immunogen acetylated tubulin from *Strongylocentrotus purpuratus* sperm axonemes) was used at a dilution of 1:10,000. Mouse monoclonal anti-dopamine transporter (AB2231; EMD Millipore) was used at 1:1000 dilution.

Iron and reactive oxygen species assays

The assay for iron concentrations in cell lines was as previously described (25). Basically, a commercial kit (Abcam) was used, following the manufacturer's instructions, to measure Fe(II) and total iron from a confluent T75 cell culture flask of each cell type per assay point. Reactive oxygen species (ROS) were measured by using the fluorescent indicator CM-H2DCFDA as previously described (43). Cells were plated in 96-well plates at 75% confluency and returned to the incubator overnight. Medium was removed from test wells and replaced with 50 μl of 5 μM probe in PBS, then incubated in the dark at 37°C for 20 min. Probe was removed from the cells and replaced with 100 μl of prewarmed DMEM. Fresh Fe(II) at a final concentration of 20 μM was added to 4 wells per experiment, and fluorescence intensity was measured using a microplate reader with FluoStar Omega (BMG Labtech) at excitation and emission wavelengths of 488 and 534 nm, respectively, at times 0, 1, and 2 h. The change in fluorescence of treated cells compared to untreated cells was used as a measure of ROS generated by Fe(II) in the treated cells over time.

Statistical analysis

Statistical analyses were conducted by 2-tailed Student's *t* test, with statistical significance considered at $P < 0.05$. Data are expressed as means ± SEM.

RESULTS

Synucleins and exogenous oligomer toxicity

There has been considerable interest in the toxicity of α-syn oligomers when applied from outside the cell (44). We have previously shown that toxic oligomers of α-syn can be generated by reacting recombinant α-syn protein with copper (37). These stellate oligomers were found to be highly toxic to neuronal cells compared to fibrils or oligomers prepared in the absence of copper. We therefore wished to further understand the mechanism of action of these stellate oligomers. We looked at the impact of increased cellular expression synuclein expression on the toxicity of recombinant α-syn oligomers applied exogenously. For clarity, the toxic α-syn oligomers will be referred to as α-syn-TO.

Stable cell lines overexpressing 1 of the 3 main synucleins were prepared by transfection of SH-SY5Y cells with pCDNA3.1 containing the ORF of human α-syn, β-syn, or γ-syn. The increased expression was confirmed by Western blot analysis as previously described (42). The stable cell lines were then treated with wild-type (WT) α-syn-TO at various concentrations. Survival of the treated cells was then determined. As can be seen in **Fig. 1*A***, α-syn-TO was toxic to SH-SY5Y cells transfected with the empty vector (pCDNA3.1) in a concentration-dependent manner. In comparison, cells overexpressing γ-syn showed no significant difference in the response to α-syn-TO compared to the controls. However, cells overexpressing α-syn were significantly more sensitive to α-syn-TO at concentrations between 0.5 and 5.0 μM, while cells overexpressing β-syn were more resistant to the toxicity at 5.0 μM and above. These results suggest that the cellular levels of both α- and β-syn influence the toxicity of α-syn-TO.

Increased expression of α-syn in cells resulted in increased sensitivity to α-syn-TO. We wished to determine if this effect could be altered by mutations in α-syn. We therefore produced stable cell lines expressing a range of structural mutations of α-syn. α-Syn-TO was applied to these cell lines at a range of concentrations in parallel with cells expressing WT α-syn or the empty vector (pCDNA3.1) as previously described (Fig. 1*B*). The mutations included a point mutation of the 1 histidine to an alanine (H50A). This mutation had no significant effect on the toxicity of α-syn-TO compared to the effect on cells expressing WT α-syn. We also included deletion mutations of both the N- and C-terminus. Deletion of the C-terminus (1–100) also had no significant effect on the toxicity of α-syn-TO. In contrast, deletion of the N terminus (Δ2–9) did have significant effect on the toxicity of α-syn-TO compared to the effect on cells expressing WT

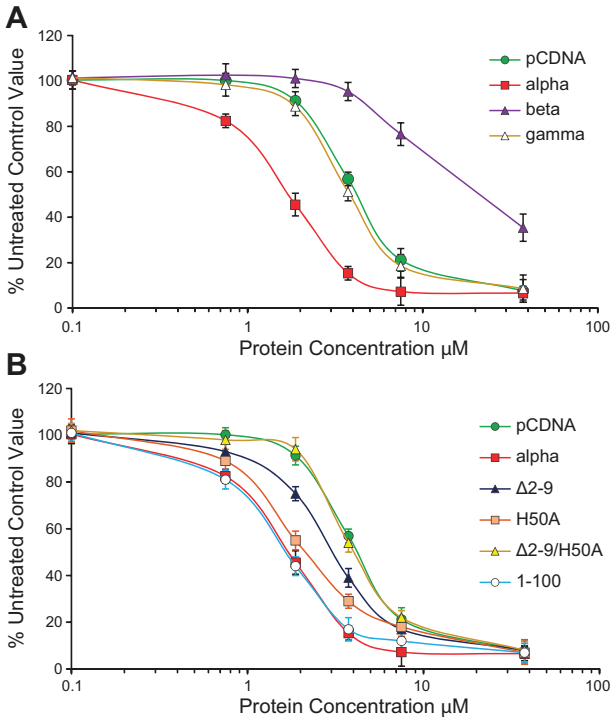

Figure 1. Toxicity of α-syn-TO to SH-SY5Y cell lines. *A*) SH-SY5Y cells overexpressing α-syn (alpha), β-syn (beta), or γ-syn (gamma) were grown in parallel with SH-SY5Y transfected with empty vector control (pCDNA). Cells were treated for 48 h with varying concentrations of recombinant α-syn-TO. Next cells were treated with MTT, and survival was assessed relative to untreated control. Cells overexpressing γ-syn showed no significant difference ($P > 0.05$, Student's t test) compared to control cells. However, cells overexpressing α-syn showed significantly more cell loss ($P < 0.05$) at concentrations between 0.75 and 4 μM compared to controls. In contrast, α-syn-TO was less toxic to β-syn–overexpressing cells at concentrations of 4 μM and higher. Shown are means ± SEM for 4 experiments with 3 replicates for each value per experiment. *B*) SH-SY5Y cells overexpressing either WT α-syn (alpha) or various mutants of α-syn were grown in parallel with SH-SY5Y transfected with empty vector control (pCDNA). Cells were treated for 48 h with varying concentrations of recombinant α-syn-TO. Next cells were treated with MTT, and survival was assessed relative to untreated control. Two mutants (H50A and 1–100, the latter mutant of α-syn or β-syn lacking residues after 100) showed no significant difference from WT α-syn in terms of sensitivity to α-syn-TO toxicity. Both Δ2–9- and Δ2–9/H50A-overexpressing cells were both significantly less sensitive to α-syn-TO toxicity than WT α-syn–overexpressing cells at concentrations between 0.75 and 4 μM. Δ2–9/H50A-overexpressing cells were not significantly different than pCDNA cells in terms of their sensitivity. This suggests that this mutant abolishes impact of increased α-syn expression on toxicity of α-syn-TO to cells. Shown are means ± SEM for 4 experiments with 3 replicates for each value per experiment.

α-syn. It was reduced for concentrations 0.5 and 5.0 μM. However, the toxicity at these concentrations was still significantly higher than the toxicity to control cells (pCDNA). Last, we also tested the toxicity of α-syn-TO on cell expressing a double mutation of both Δ2–9 and H50A (Δ2–9/H50A). In this case, the toxicity of α-syn-TO to these cells was not significantly different than that of the toxicity to control cells. This implies that the mutation Δ2–9/H50A abolished the effect of overexpressed α-syn that increased cell sensitivity to α-syn-TO toxicity.

We also wished to assess whether similar structural changes would alter the toxicity of α-syn-TO. We therefore generated recombinant protein for α-syn and generated aggregated protein through the same method as WT α-syn. The mutant forms of α-syn-TO generated were applied to SH-SY5Y cells for 48 h, and the viability measured and compared to the toxicity of WT α-syn-TO (**Fig. 2*A***). A deletion from the C-terminus (Δ119–126) had no significant effect on the toxicity of α-syn-TO. However, the point mutation H50A significantly reduced toxicity at concentrations between 2.0 and 7.5 μM, but not at higher concentrations. In contrast, the N-terminal mutation Δ2–9 abolished toxicity. We assessed the ability of these mutations to form aggregates using a ThT assay (Fig. 2*B*). The ThT assay verified that all proteins used in these studies were able to form aggregates. Therefore, the differences in toxicity were not due to differences in ability to aggregate.

α-Syn and FoxO3a

FoxO3a is a transcription factor associated with cell death in neurodegenerative diseases. There is good evidence that increased expression of active FoxO3a increases neuronal loss in transgenic rats overexpressing α-syn (41). We wished to determine if the increased cell loss seen in our model was related to a change in FoxO3a expression. We grew SH-SY5Y cells either overexpressing WT α-syn or its mutant Δ2–9/H50A and control cells transfected with the empty vector (pCDNA3.1). Protein extracts were prepared from the cells, and Western blot analysis and immunodetection were performed. Blots were analyzed for the expression of total FoxO3a, p-FoxO3a, and tubulin as a loading control (**Fig. 3**). The results showed that total FoxO3a but not p-FoxO3a were elevated in cells overexpressing WT α-syn but not the mutant Δ2–9/H50A. The lack of altered expression of the phosphorylated form is indicative that the change in expression is not due to an increase in the inactive form of FoxO3a. The lack of increased expression of FoxO3a in the mutant Δ2–9/H50A as opposed to WT α-syn correlated with their relative susceptibility to α-syn-TO toxicity.

FoxO3a is a transcription factor. As such, it acts *via* binding to DNA. Inhibition of FoxO3a activity can be achieved through prevention of DNA binding. We therefore created SH-SY5Y cell lines that overexpressed either WT FoxO3a (FoxO3a-WT) or the DNA-binding domain (DBD) of FoxO3a. Either the ORF of FoxO3a or the DNA binding domain (encoding 139 aa without the transactivation domain, residues 138–277) were cloned into pCDNA3.1. Both constructs also include a hemagglutinin tag for easy detection of the overexpressed protein. Cells were transfected with the constructs and stable cell lines selected by exposure to G418. Once overexpression in the cells was verified (data not shown), the cell lines and controls were treated with WT α-syn-TO and survival assessed after 48 h (**Fig. 4**). Cells overexpressing FoxO3a-WT showed a slight but significant increase in susceptibility

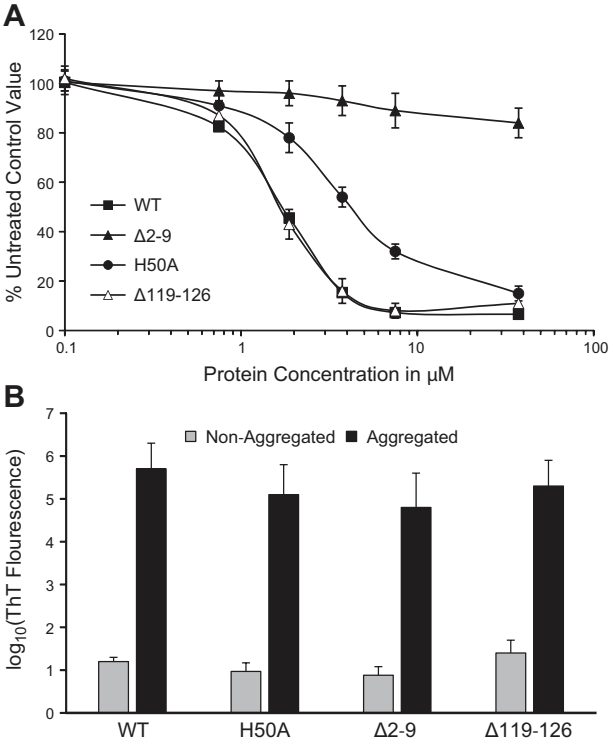

Figure 2. Toxicity of α-syn-TO mutants. *A*) Purified recombinant α-syn was generated and used to form α-syn-TO. As well as WT α-syn (WT), mutant forms of α-syn were also expressed and purified to generate α-syn-TO with different mutations. These mutants included N-terminal (Δ2–9) and C-terminal mutant (Δ119–126), and single-point mutation (H50A). Cells were treated for 48 h with varying concentrations of different recombinant α-syn-TOs in parallel. Next cells were treated with MTT, and survival was assessed relative to untreated control. Δ119–126 mutation had no significant effect on α-syn-TO toxicity compared to WT. In contrast, both H50A and Δ2–9 reduced α-syn-TO toxicity significantly at concentrations between 2 and 7.5 μM. Δ2–9 mutant showed no significant difference between different concentrations, suggesting it was not toxic at concentrations tested. Shown are means ± SEM for 4 experiments with 3 replicates for each value per experiment. *B*) Mutations of α-syn used in experiments were tested for their ability to form β-sheets as determined by ThT assay. Purified protein of different mutants and WT α-syn were shaken to generate α-syn-TO. After 7 d, samples of aggregated proteins were taken and reacted with ThT along with samples of protein that had not been subjected to shaking (nonaggregated). Fluorescence at 482 nm was determined for all samples. No significant difference ($P > 0.05$) was seen for aggregated protein of different mutants compared to aggregation of WT α-syn-TO. Shown are means ± SEM of 4 different experiments.

to α-syn-TO toxicity compared to toxicity to pCDNA control cells. In contrast, cells overexpressing FoxO3a-DBD were strongly and significantly protected from α-syn-TO toxicity. These results suggest that FoxO3a plays an important role in cell death caused by α-syn-TO. These results suggest that elevated FoxO3a expression induced by α-syn is the cause of the increased susceptibility of SH-SY5Y cells to α-syn-TO toxicity.

FoxO3a and iron reduction by α-syn

Having established a role for FoxO3a in cell death induced by α-syn-TO, and having also established that increased expression of FoxO3a is related to increased expression of α-syn, it is important to try and identify how α-syn increases FoxO3a expression. We have shown previously that α-syn possesses ferrireductase activity and causes an increase in cellular reduced iron [Fe(II)] (25). Therefore we set out to determine if altering iron levels in cells would alter FoxO3a expression. SH-SY5Y cells were treated with 50 μM Fe(II) in serum-free conditions for 24 h. Extracts were then prepared from the cells and untreated controls, and Western blot analysis for FoxO3a levels was carried out (**Fig. 5*A***). Treatment with Fe(II) resulted in a significant increase in FoxO3a expression compared to controls. Additionally, we treated α-syn–overexpressing cells with the iron chelator deferiprone (250 μM) for 24 h under serum-free conditions. Deferiprone is a strong, cell-permeable Fe(II) chelator. We then measured the level of expression of FoxO3a using Western blot analysis (Fig. 5*B*). Deferiprone significantly decreased FoxO3a expression compared to

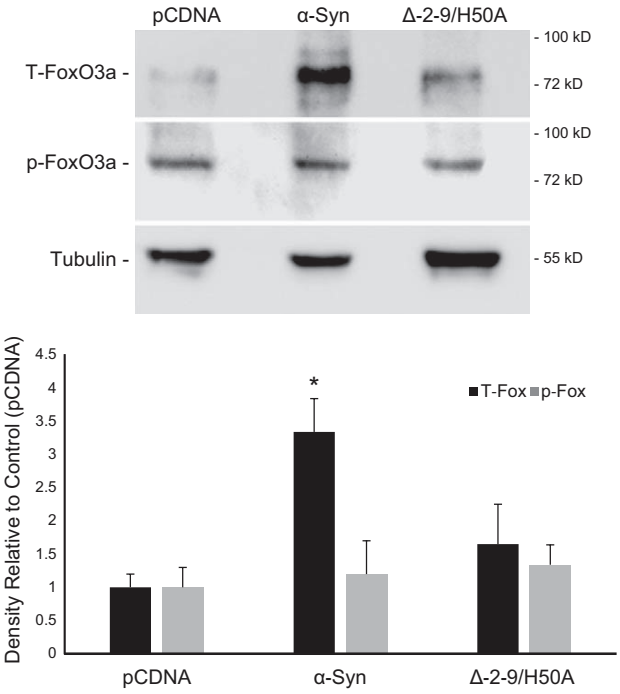

Figure 3. FoxO3a and α-syn expression. Protein extracts were prepared from SH-SY5Y cells overexpressing either WT α-syn or α-syn mutant Δ2–9/H50A as well as cells transfected with empty vector (pCDNA). Protein extracts were electrophoresed on PAGE gels and Western blot analysis performed. Specific antibodies were used to detect total FoxO3a (T-FoxO3a), p-FoxO3a, α-syn, and tubulin. α-Syn detection was included to verify overexpression of protein. Densitometric analysis was then performed for FoxO3a bands and normalized to tubulin. Average values and SEM for $n = 4$ blots were determined and plotted relative to levels of pCDNA. Statistically significant ($P < 0.05$) increase was observed for total FoxO3a in α-syn–overexpressing cells but not for p-FoxO3a, and no significant change was observed for either in Δ2–9/H50A cells.

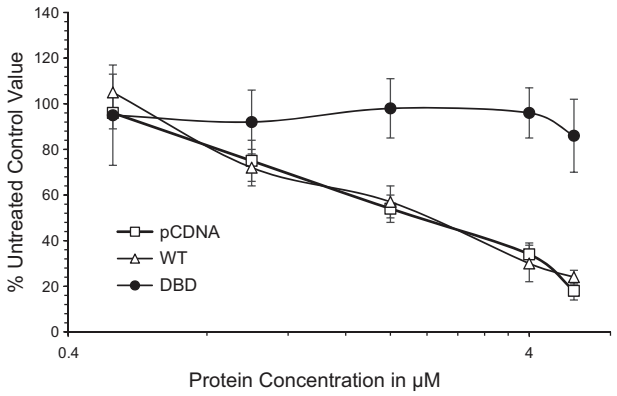

Figure 4. Toxicity of α-syn-TO and FoxO3a. SH-SY5Y cells overexpressing either WT FoxO3a (WT) or just DBD along with control cells (pCDNA) were treated for 48 h with varying concentrations of recombinant α-syn-TO. Next cells were treated with MTT, and survival was assessed relative to untreated control. Increased expression of WT FoxO3a had no effect on survival of cells compared to empty vector. In contrast, DBD-overexpressing cells showed significant ($P < 0.05$) difference from controls at 1.0 μM and above. Presence of DBD showed high levels of protection from α-syn-TO toxicity. Shown are means ± SEM for 4 experiments with 3 replicates for each value per experiments.

controls cells treated only with the vehicle (DMSO). These results suggest that the levels of Fe(II) might alter the expression of FoxO3a.

We wished to test whether another ferrireductase could alter FoxO3a expression. A known mammalian ferrireductase, Steap3 (45), was cloned into pCDNA3.1 and overexpressed in SH-SY5Y cells. We then determined whether total and p-FoxO3a levels were altered in these cells. In parallel, we also looked at FoxO3a expression in cells overexpressing β-syn, which has no ferrireductase activity (25). Cells overexpressing Steap3 showed significantly increased levels of total FoxO3a but reduced levels of p-FoxO3a, while those overexpressing β-syn did not (**Fig. 6**). These results suggest that overexpressing a ferrireductase can alter FoxO3a expression, but overexpression of another synuclein does not. We then tested the toxicity of α-syn-TO to Steap3-overexpressing cells (**Fig. 7*A***). Compared to the survival of control cells, α-syn-TO was more toxic to Steap3-overexpressing cells. Therefore, another known ferrireductase overexpressed in cells leads to increased FoxO3a expression and increases sensitivity to α-syn-TO. This is similar to the effect of α-syn overexpression. When combining these findings with the data mentioned above on iron and FoxO3a expression, the most likely explanation for the alteration in FoxO3a levels is due to the increased levels of Fe(II) generated through iron reduction by α-syn.

β-Syn and α-syn-TO toxicity

Having elucidated a possible mechanism for how α-syn enhanced the toxicity of α-syn-TO, we wished to look further at how β-syn can have the opposite effect. We used a battery of β-syn structural mutants, which included deletion of the N terminus (Δ2–9) or the C-terminus (1–100), point mutation of the single histidine (H65A), and a double mutation of the N terminus (Δ2–9/H65A). Stable cell lines were generated overexpressing each of these mutants. The cell lines were then treated with α-syn-TO and compared to cell lines overexpressing WT β-syn of controls cells transfected with the empty vector (pCDNA). Survival of the cell lines was measured after 48 h with an MTT assay (Fig. 7*B*). Neither the C-terminal mutant or the point mutation has any significant effect on the toxicity compared to WT β-syn. However, the N-terminal mutation did significantly alter the toxicity, reducing the protective effect of β-syn. In comparison, the mutant Δ2–9/H65A had an even stronger effect. There was no significant difference in toxicity between Δ2–9/H65A and pCDNA at any point, suggesting that this mutation abolished toxicity.

We have shown that β-syn has no effect on FoxO3a levels. Therefore, it is unlikely that the protective effect of β-syn against α-syn-TO toxicity is related to altered FoxO3a levels. We have previously shown that β-syn reduced the level of Fe(II) present in cells overexpressing the protein (25). We therefore looked at the levels of Fe(II) in both β-syn–overexpressing cells and the mutant Δ2–9/H65A, which has no protective effect against α-syn-TO toxicity. **Figure 8*A*** shows that overexpression of β-syn decreases Fe(II) levels in cells but that overexpression of Δ2–9/H65A has no significant effect. The presence of Fe(II) in cells is known to potentially increase generation of ROS. Therefore, we also measured ROS levels in cells with and without exposure to exogenous Fe(II). Overexpression of β-syn caused a decrease in the levels of ROS detected in SH-SY5Y cells, while overexpression of the mutant Δ2–9/H65A did not (Fig. 8*B*). Therefore, the protective effect of β-syn against α-syn-TO toxicity may be due to a reduction in cellular sensitivity to ROS generated by Fe(II). Because α-syn is a known ferrireductase (32) that increases Fe(II) levels in cells, this fits well with β-syn's known role as antagonistic to pathologic changes caused by α-syn.

DISCUSSION

The research presented here provides evidence for significant advancements in the understanding of the toxicity of oligomeric species of α-syn. First, toxicity is mediated through increased activity of FoxO3a. This protein is strongly associated with cell-death pathways and has been previously shown to be increased in the Lewy bodies of patients with PD (40). Second, increased expression of α-syn causes increased sensitivity to oligomer toxicity. This is likely due to increased expression of FoxO3a in the α-syn–overexpressing cells. Third, increased β-syn expression has a protective effect against oligomer toxicity. This fits well with numerous reports that β-syn has a protective role against the negative effects of increased α-syn expression. This may be a result of interaction with α-syn (46–49) or other means (50).

The involvement of FoxO3a in cell death in α-syn overexpression has been demonstrated in a transgenic rat

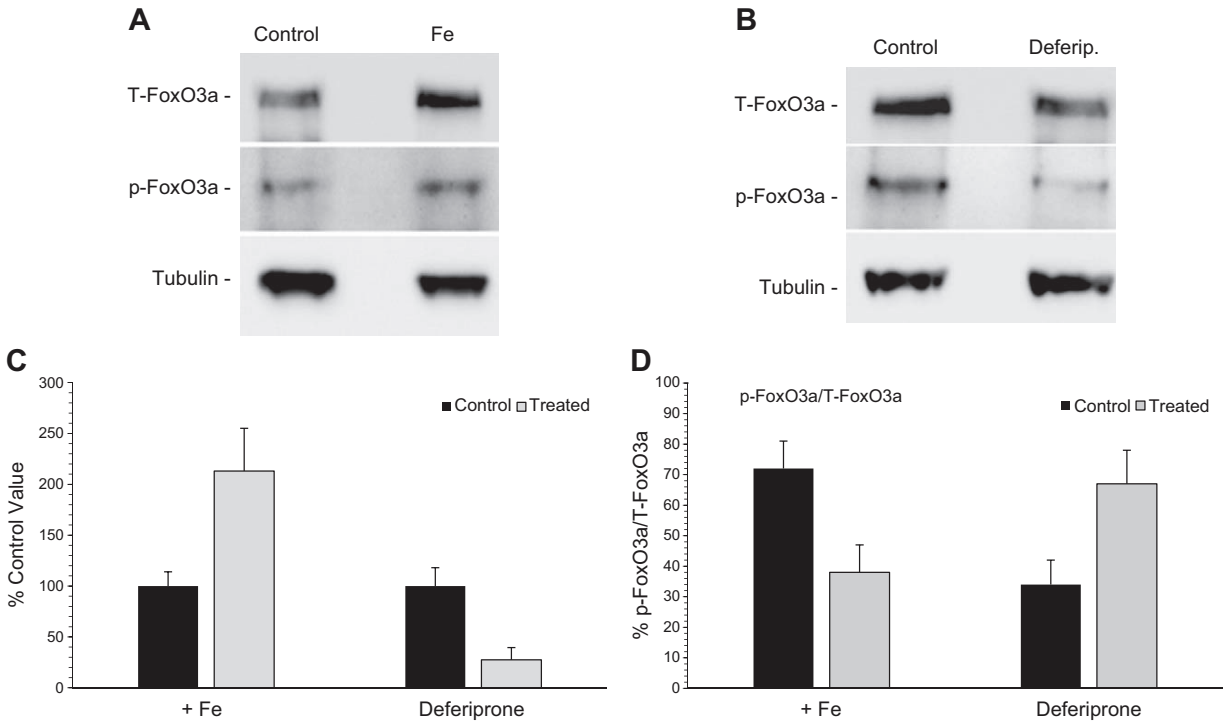

Figure 5. Iron and FoxO3a expression. *A*) SH-SY5Y cells were treated with 50 μM Fe(II) for 24 h. Protein extracts were prepared from treated cells (Fe) and untreated controls. Protein extracts were electrophoresed on PAGE gels and Western blot analysis performed. Specific antibodies were used to detect total FoxO3a (T-FoxO3a), p-FoxO3a, and tubulin. Densitometric analysis was then performed for FoxO3a bands and normalized to tubulin. *B*) SH-SY5Y cells overexpressing α-syn were treated with 250 μM deferiprone for 24 h. Protein extracts were electrophoresed on PAGE gels and Western blot analysis performed. Specific antibodies were used to detect total FoxO3a (T-FoxO3a), p-FoxO3a, α-syn, and tubulin. Densitometric analysis was then performed for FoxO3a bands and normalized to tubulin. *C*) Changes in total FoxO3a for treatments with iron and deferiprone are shown as percentage of control value for each experiment. Fe treatment significantly increased levels of total FoxO3a in SH-SY5Y cells. In contrast, deferiprone significantly ($P < 0.05$) decreased expression of FoxO3a in α-syn–overexpressing cells. Shown are means ± SEM of 4 different experiments. *D*) Ratio of p-FoxO3a to T-FoxO3a was also determined. Densitometric values were normalized to tubulin; then value for p-FoxO3a was divided by value for T-FoxO3a for each group and converted to percentage. Treatment with Fe significantly decreased ratio of p-FoxO3a/T-FoxO3a despite overall increase in T-FoxO3a. In contrast, deferiprone treatment led to significant ($P < 0.05$) increase in ratio of p-FoxO3a/T-FoxO3a. Shown are means ± SEM of 4 different experiments.

model (41). In this model, pathologic effects of α-syn transgenic overexpression are reversed by inhibition of FoxO3a activity. This inhibition was induced by coexpression of the dominant negative inhibitor of FoxO3a activity, namely the DNA binding domain of FoxO3a. Our results support this role, as we used a similar construct, overexpressed it in SH-SY5Y cells, and showed that this inhibited the toxicity of α-syn toxic oligomers. In contrast, overexpressing WT FoxO3a had no such effect. In this case, it is possible that the extra FoxO3a did not increase the amount of active (nonphosphorylated) FoxO3a that entered the nucleus. Alternatively, a second factor may also be necessary for α-syn-TO toxicity, which would remain limiting despite the increased levels of FoxO3a. Either way, these data suggest that FoxO3a activity is necessary for α-syn-TO toxicity.

FoxO3a expression in SH-SY5Y cells was shown to be increased by the overexpression of α-syn or Steap3 but not β-syn. A mutant of α-syn (Δ2–9/H50A) also had no effect on FoxO3a expression. This implies that the mutation alters α-syn sufficiently so that its overexpression no longer affects the expression of FoxO3a. In each case, increased FoxO3a expression was not accompanied by increased p-FoxO3a expression, confirming that the increase is not just elevated accumulation of inactive FoxO3a (51). The increased expression of FoxO3a correlated with higher susceptibility of SH-SY5Y cells to α-syn-TO toxicity. In other words, overexpression of α-syn and Steap3 both increased FoxO3a expression and susceptibility to α-syn-TO toxicity, but for Δ2–9/H50A and β-syn there was no increase in FoxO3a and no observed increase in α-syn-TO toxicity. These results also support the suggestion that FoxO3a expression mediates the toxicity of α-syn-TO. These findings, in combination with previous work in transgenic rats, imply that FoxO3a is of major significance in terms of neuronal death mediated by α-syn.

It is also important to consider the mechanism by which FoxO3a activity or expression is increased. Both α-syn and Steap3 show ferrireductase activity, while Δ2–9/H50A and β-syn do not (25, 45). The implication is that the

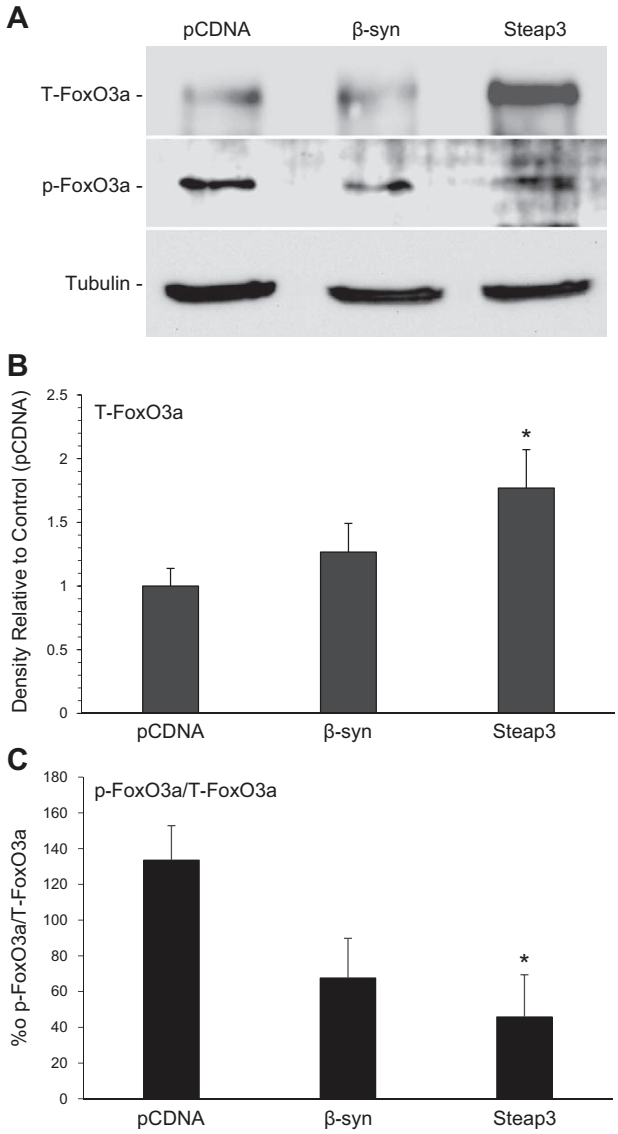

Figure 6. FoxO3a expression and ferrireduction. *A*) Protein extracts were prepared from SH-SY5Y cells overexpressing either Steap3 or β-syn as well as from cells transfected with empty vector (pCDNA). Protein extracts were electrophoresed on PAGE gels and Western blot analysis performed. Specific antibodies were used to detect total FoxO3a (T-FoxO3a), p-FoxO3a, and tubulin. *B*) Densitometric analysis was then performed for total FoxO3a bands and normalized to tubulin. *C*) Ratio of p-FoxO3a to T-FoxO3a was also determined. Shown are means ± SEM of 4 different experiments. *Values for Steap3, but not β-syn are significantly different from control ($P < 0.05$).

common factor resulting in increased FoxO3a expression is the generation of elevated levels of Fe(II), the product of reactions catalyzed by ferrireductases. We verified this finding by demonstrating that increased iron caused an increase in FoxO3a expression in SH-SY5Y cells. The relative change in p-FoxO3a was much less, thus implying that the increase was active FoxO3a. We showed the relevance of this finding for our system by applying an iron chelator to SH-SY5Y cells overexpressing α-syn and observing a reduced expression of FoxO3a. The change resulted in a high level of p-FoxO3a compared to the total, indicating the reduction was in active FoxO3a. Therefore, the fundamental factor that both regulates FoxO3a and susceptibility of SH-SY5Y cells to the toxicity of α-syn-TO may be Fe(II).

While our findings are novel, there have been a number of studies relating increased iron levels with increased activity of FoxO-family transcription factors (52, 53). One

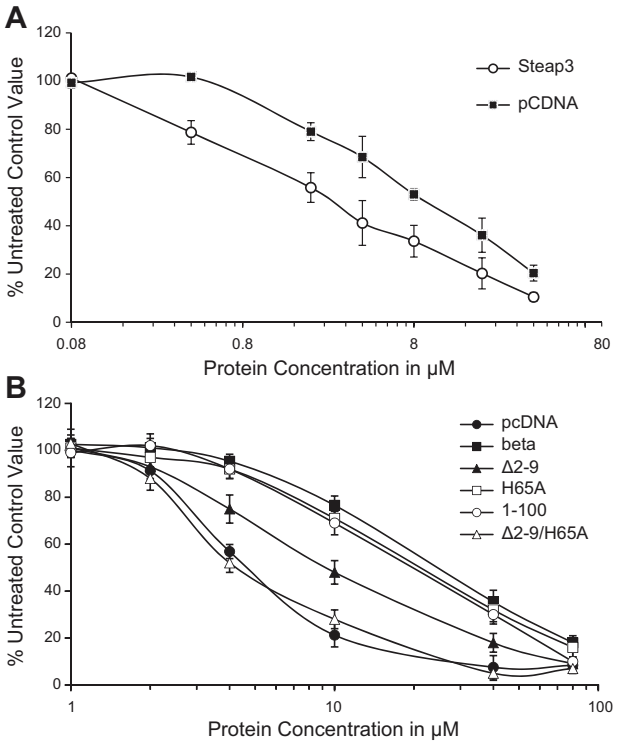

Figure 7. α-Syn-TO toxicity to β-syn–expressing cell line. *A*) SH-SY5Y cells overexpressing Steap3 were grown in parallel with SH-SY5Y transfected with empty vector control (pCDNA). Cells were treated for 48 h with varying concentrations of recombinant α-syn-TO. Next cells were treated with MTT, and survival was assessed relative to untreated control. Steap3 cells showed significantly ($P < 0.05$) greater sensitivity to toxicity of α-syn-TO at concentrations between 2 and 8 μM compared to pCDNA cells. Shown are means ± SEM for 4 experiments with 3 replicates for each value per experiment. *B*) SH-SY5Y cells overexpressing either WT β-syn (alpha) or various mutants of α-syn were grown in parallel with SH-SY5Y transfected with empty vector control (pCDNA). Cells were treated for 48 h with varying concentrations of recombinant α-syn-TO. Next cells were treated with MTT, and survival was assessed relative to untreated control. Two mutants (H65A and 1–100, the latter mutant of α-syn or β-syn lacking residues after 100) showed no significant difference from WT β-syn in terms of sensitivity to α-syn-TO toxicity. Both Δ2–9- and Δ2–9/H65A-overexpressing cells were significantly ($P < 0.05$) less sensitive to α-syn-TO toxicity than WT β-syn–overexpressing cells at concentrations between 4.0 and 40 μM. Δ2–9/H65A-overexpressing cells were not significantly different than pCDNA cells in terms of their sensitivity. This suggests that this mutation abolishes impact of increased β-syn expression on protection from toxicity of α-syn-TO to cells. Shown are means ± SEM for 4 experiments with 3 replicates for each value per experiments.

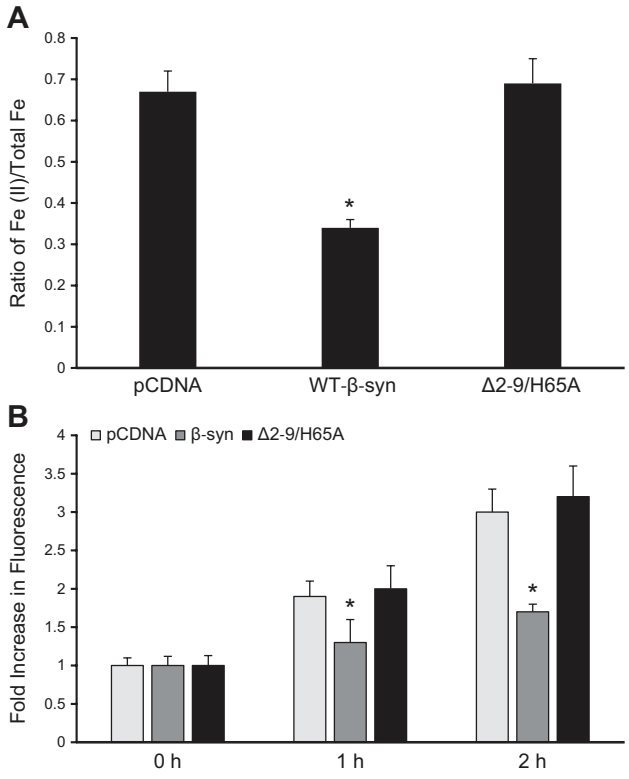

Figure 8. β-Syn– and iron-induced ROS. *A*) Ratio of Fe(II) to total Fe within cells was determined for SH-SY5Y cells overexpressing either β-syn or mutant of β-syn, Δ2–9/H65A, and compared to cells transfected with empty vector using commercial kit. Shown are means ± SEM for 4 experiments. *Values for β-syn were significantly different ($P < 0.05$) than for pCDNA. *B*) Levels of ROS in same cell lines were determined by fluorescent compound CM-H2DCFDA. Cells loaded with CM-H2DCFDA were treated with 20 μM Fe(II) for 0, 1, or 2 h. Measurements were made of cells with and without treatment with Fe(II). Increase in ROS detected in Fe(II)–treated cells above that of untreated cells was measured and plotted as fold increase. As can be seen, Fe(II) increased ROS levels in all cell lines when comparing time points. Increase in ROS detected in β-syn–overexpressing cells was significantly lower ($*P < 0.05$) than in other cell lines at both 1- and 2-h time points. Shown are means ± SEM for 3 experiments.

such study implies that the increase of FoxO3a is mediated by the PI3K/AKT pathway. It has also been shown that iron overload increases FoxO3a expression (54). It is well known that FoxO3a expression is increased under oxidative stress and that Fe(II) is readily able to catalyze reactions that increase oxidative stress such as the Fenton reaction (55). Iron-induced oxidative damage has frequently been shown to be mediated through the PI3K/AKT pathway (56–58). Therefore, it is possible that the increased active FoxO3a seen in our findings is a result of down-regulation of the PI3K/AKT pathway caused be oxidative stress from Fe(II).

These findings suggest that elevated cellular Fe(II) caused by overexpression of α-syn increases cellular susceptibility to the toxicity of oligomers of exogenous α-syn. Previous studies have also provided evidence that iron and α-syn can act in concert to cause cell death (59, 60). This is of considerable interest because of the possible role of this mechanism in diseases such as PD. Neuronal loss in the *substantia nigra* is the hallmark of the disease. Patients with PD show both elevated levels of α-syn and increased levels of Fe(II) (61). There is a long history of the relation of altered iron levels and PD, but there has never been a causal connection established between elevated iron and loss if dopaminergic neurons in PD (62–67). However, a study in transgenic rodents suggested that iron chelation reduced pathologic changes caused by α-syn overexpression (68), and another study suggested that iron chelation also protected against the toxicity of 6-hydroxy dopamine in a mouse model (69).

Ambivalence exists as to whether α-syn is genuinely toxic *in vivo*, and the form of the toxic species is also in question. There is strong evidence that α-syn can be toxic, especially when overexpressed or introduced exogenously (70, 71). For some time toxicity was thought to come from fibrils, but this has largely been dismissed in favor of the "toxic oligomer" hypothesis (72). There are many contenders for the mechanism of action of these (44). We developed a method to generate highly toxic oligomers by reacting recombinant α-syn with copper during a shaking process (37). The oligomers generated were stellate, and their toxic action was not dependent on the copper required for their formation. However, there are other kinds of oligomers, such as the pore-forming variety (73, 74). While no one could claim to have generated the single toxic oligomer that best models an *in vivo* oligomer, we think our oligomer model is a good one because of its unique nature, reproducibility of toxic profile, high toxicity, and ability to induce changes seen *in vivo* (such as those described in this report).

We showed in our data that β-syn overexpression protects against the toxicity of α-syn oligomers. Because this is the opposite of the effect of overexpression of α-syn, it further emphasizes the potential role of β-syn as a regulator of the activity of α-syn. Expression of both α-syn and β-syn is regulated by similar pathways and transcription factors (75). This system probably exists to ensure that levels of the two proteins are in balance. This balance would then protect against problems such as aggregation and toxicity of α-syn. Expression patterns and levels of α-syn and β-syn most closely overlap (3). β-Syn is the most abundantly expressed synuclein in the brain, comprising 75% to 80% of the total mRNA of the synucleins (76). In both the mouse brain and the human *substantia nigra*, α-syn mRNA decreases and β-syn mRNA increases with age (77). In contrast to control patients, there is a dramatic increase in α-syn and a decrease in β-syn mRNA levels in the *substantia nigra* of PD, diffuse Lewy body disease, and a Lewy body variant of AD (76). The importance of the balance between levels of the synucleins is highlighted by the observation that β-syn inhibits α-syn aggregation *in vitro* and *in vivo* (78–80). In addition, β-syn prevents aggregated α-syn from inhibiting the 26S proteasome (81). These functions lead to expression of β-syn in transgenic α-syn mouse models ameliorating neurodegenerative alterations, decreasing Lewy body formation and preventing motor deficits (78, 82).

The nature by which β-syn exerts these effects is not understood. However, it is possible that it occurs through direct interactions. Both synucleins are able to form dimers and tetramers (83, 84), and the latter is believed to be the correct form for cellular α-syn. Therefore, it is possible that heterotetramers exist that include both synucleins and that lack activities such as the proposed ferrireductase activity (25). We have previously demonstrated that cells overexpressing β-syn have lower Fe(II) levels than control cells (25). It is possible that this reduced level of Fe(II) protects cells when exposed to α-syn-TO. However, this is not reflected in a change in FoxO3a, as levels were not reduced in cells overexpressing β-syn. We showed that a mutant form of β-syn (Δ2–9/H65A) did not have the same effect as WT β-syn in protecting cells from α-syn-TO toxicity. While cells overexpressing β-syn showed both lower levels of intracellular Fe(II) and Fe(II)-generated ROS, Δ2–9/H65A did not. This may be because it is unable to interact with α-syn or is unable to be incorporated in synuclein tetramers. We have shown that membrane associated tetramers of α-syn are the form that has iron-reducing activity (32). If the mutant Δ2–9/H65A has a reduced ability to inhibit this activity, then cells would generate more Fe(II). As we have shown, Fe(II) can increase FoxO3a levels, which mediates the toxicity of α-syn-TO.

In summary, we have demonstrated that FoxO3a plays a pivotal role in the toxicity of α-syn oligomers. Inhibition of its activity blocks toxicity. Overexpression of α-syn increases Fe (II) levels in cells, which then increases FoxO3a levels, leading to elevated cellular sensitivity to oligomer toxicity. In contrast, β-syn overexpression decreases Fe(II) levels and decreases cellular sensitivity to toxic oligomers. We believe these findings provide an insight into the mechanism of dopaminergic neuronal loss in the synucleinopathies. We suggest that FoxO3a and increased Fe(II) levels are key factors in the pathway that leads to cell death. FJ

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AUTHOR CONTRIBUTIONS

D. M. Angelova, H. B. L. Jones, and D. R. Brown performed the experiments; and D. R. Brown designed the experiments and wrote the article.

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4.5 Concluding commentary

In the chapter above, we presented a potential mechanism through which elevated α-synuclein can contribute to neuronal death. According to our results increased α-synuclein expression results in increased ferrireductase activity and thus elevated levels of Fe^{2+}. Elevated Fe^{2+} caused oxidative stress and triggered the activation of the FOXO3a pathway leading to cell death. The data suggested that it was the ferrireductase activity of α-synuclein that elicited these effects as they were replicated by the overexpression of another ferrireductase – STEAP3. β-synuclein on the other hand did not replicate the effect α-synuclein had and in fact overexpressing it resulted in lower levels of iron and ROS, suggesting that β-synuclein was protective against toxicity.

This finding together with the studies presented in previous chapters point to one thing – that increased iron levels can replicate the age-related changes seen in microglia and that those changes can influence the proteins directly linked to the pathology of neurodegenerative disease (α-synuclein and β-amyloid). All work described in the above chapters will be discussed in detail in the next chapter (Discussion).

5. Discussion

5.1 Summary of results

The major findings of this thesis can be summarised as follows:

Chapter 2 presented and characterised a novel aged microglia model by using the human microglial cell line SV40. It was found that their morphology changed to a less ramified and more amoeboid one when treated with iron. They had elevated iron storage and increased ferritin expression. Iron-treatment did not affect their levels of proliferation. The expression of some inflammatory cytokines was altered by iron treatment, namely IL-1β, IL-6 and IL-8 similarly to aged microglia while others such as TNFα, IL-2, IL-10 and IL-13 were unchanged. Iron-fed microglia also showed changes in other aged microglia markers such as decreased glutamate release, decreased SIRT1 expression and increased KV1.3 expression.

Conditioned medium from the iron-fed human microglia cell line was used to investigate the effects of iron-fed microglia on APP processing in SHSY5Y cells. It was found that reduced levels of IDE in the conditioned medium resulted in reduced β-amyloid degradation. The reduced secretion of IDE was due to ER stress induced by the elevated iron and the resultant reduction in autophagic flux in the iron-fed microglia.

Chapter 3 characterised the aged microglia model using the mouse microglial cell line C8B4 and mouse primary microglial cells. C8B4 and primary microglia showed a loss in projections and C8B4 showed deramification and membrane fragmentation. Iron-fed C8B4 microglia origin were found to store significantly higher levels of iron and express more ferritin but not to release that iron into their conditioned medium. Their proliferation and cell death rates were found to be lower than control cells. Iron-fed microglia also exhibited increased expression of KV1.3 and increased secretion of reactive oxygen species. The levels of inflammatory cytokines released by iron-fed C8B4 and primary microglia were also found to change, where TNFα consistently elevated and IL-6 decreased in both cell types. Other cytokines such as IL-1β were elevated in C8B4 microglia and decreased in primary microglia, while IL-10 showed an increase in primary microglia and decreased in C8B4. The chemokine KC-GRO was only detected in primary microglia and was found to be elevated.

Conditioned medium from both iron-fed C8B4 and iron-fed primary microglia was found to increase α-synuclein levels in SHSY5Y cells. That was probably mediated by a transcription factor that bound the promoter of α-synuclein. This increase was most likely caused by the elevated levels of TNFα in the iron-fed microglia conditioned medium and mediated through the NFκB pathway. The effects of iron-fed microglia seen in Chapter 2 and 3 are summarised in figure 5.1.

Chapter 4 found that elevated levels of α-synuclein in SHSY5Y cells increased the toxicity of α-synuclein toxic oligomers. This was most likely mediated through the activity of

FOXO3a. The elevation in FOXO3a expression was found to be caused by increased levels of ROS. The elevated levels of ROS were generated by an increased level of Fe^{2+} which is a product of the ferrireductase activity of α-synuclein.

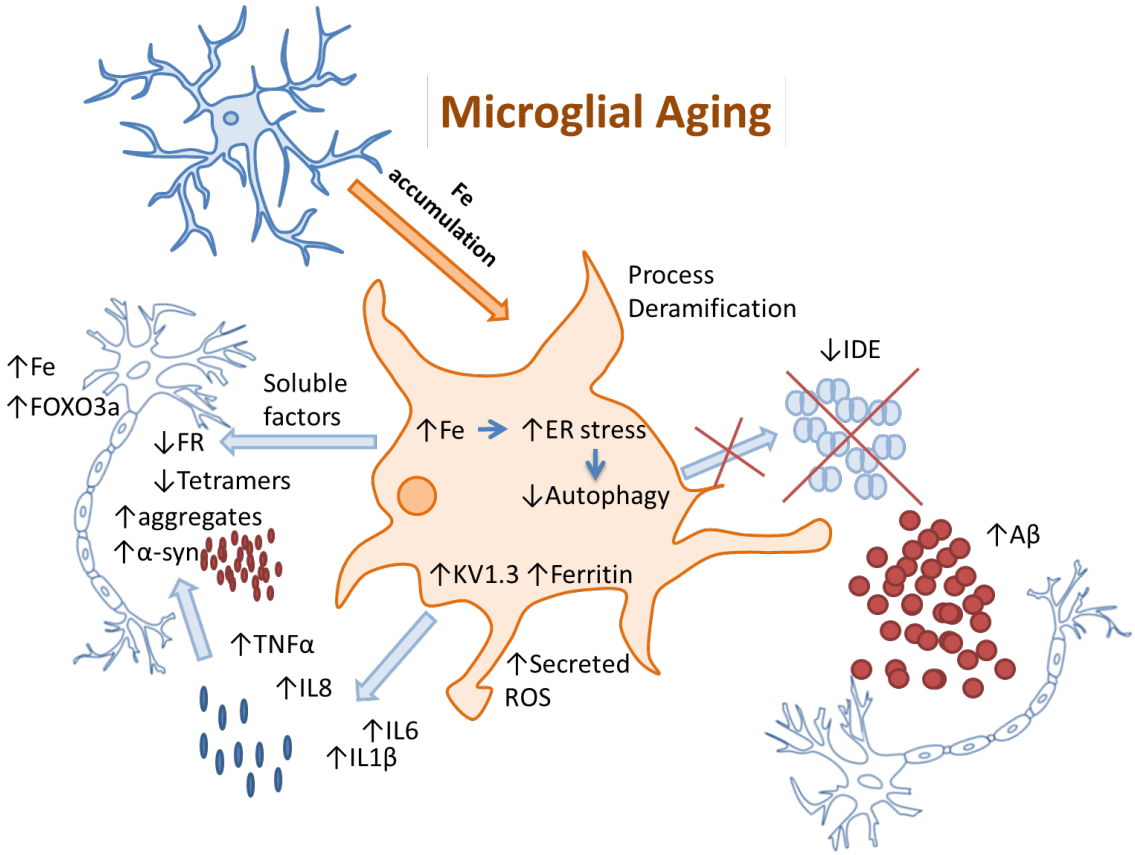

Figure 5.1. Microglial aging model induced by iron. This figure summarises the effects caused by iron accumulation in aging microglia. It leads to an altered microglial morphology and altered intracellular processes namely increased ER stress leading to decreased autophagy. That leads to decreased release of IDE which results in decreased degradation of β-amyloid in the extracellular space. Iron accumulation also leads to increased secretion of inflammatory cytokines and ROS from which TNF-α causes increases in α-synuclein levels and toxicity in neurons mediated through FOXO3a.

5.2 Comparison of the different iron-fed microglial models

The treatment of microglia with iron resulted in a largely similar phenotype in all three models, namely: increased iron storage, a more deramified morphology, and generation of more proinflammatory cytokines. However, some discrepancies between the different cell lines and between cell lines and primary cells were noted.

The first difference of note was the proliferation rates of iron-fed microglia compared to untreated. The proliferation of the iron-fed human microglial cell line was not found to change while in the C8B4 mouse microglial cell line they were found to proliferate significantly slower. Proliferation of cells in culture is generally controlled by tumour suppressor proteins such as pRb, p53, p16 and p27(Rayess et al. 2012; Kossatz and Malek 2007; Giacinti and Giordano 2006; Harris 1996). It can also be activated by mitogens such as EGF or the activation of MAP kinase (Sontag et al. 1993; Hebert et al. 2009). Microglial proliferation is a slow process but its rate can be increased by inflammatory signals such as IL-1β and TNFα but also hydrogen peroxide (Mander et al. 2006). In the cell lines used in this project proliferation was induced artificially or spontaneously and it's possible that the effects of these external interventions could have obscured any expected changes in proliferation. Yet, that still doesn't explain how the iron treatment could have induced different effects on the different cell lines. The human cell line was immortalized by transfection with the SV40 viral vector. SV40 can inactivate tumour suppressor pathways such as p53 and pRb and also induce telomerase activity in order to prevent replicative senescence (Hubbard and Ozer 1999). C8B4 on the other hand was a spontaneously transformed cell line without the addition of any carcinogens or oncogenic viruses (Alliot et al. 1996). It is possible that iron treatment could have activated tumour suppressor pathways which was masked in the human microglial cell line but observed in C8B4 cells because their tumour suppressor pathways were not artificially inactivated. Additionally, the background rate of proliferation of the two cell lines would have affected the measurements. The human microglia cell line proliferated significantly faster than C8B4s which could have also obscured any effects on proliferation that iron may have had.

Secondly, the cytokine profile of the three cell types measured was different to begin with and in some cases reacted differently when the cells were treated with iron. Firstly, IL-1β was elevated in iron-fed cell lines but decreased in iron-fed primary microglia. Additionally, IL-6 was found to increase in human iron-fed microglia and the C8B4 mouse microglia but decreased in iron-fed primary mouse microglia. On top of that IL-10 was unchanged in human microglia, decreased in C8B4 and increased in primary microglia. TNFα was also unchanged in iron-fed human microglia but elevated in both types of mouse microglia. Finally, primary mouse microglia were found to release large amounts of the chemokine KC-GRO while C8B4 cells did not release it at all.

In terms of the studies presented in this thesis, these discrepancies raise a question about the consistency of the iron-fed microglia model. Most importantly as the human iron-fed microglia cell line didn't express significantly more TNF-α than controls it is doubtful that conditioned medium from human microglia would be able to replicate the effects seen with C8B4 or primary microglia conditioned medium such as the increased expression of α-synuclein in SH-SY5Y cells treated with conditioned medium. That could potentially also be explained through the basal difference in proliferation rates between the two cell lines.

It is possible that these rates are an autocrine system related to the sensitivity of the cells to their own cytokines which would also alter their secretory phenotype. Additionally, iron-fed primary microglia had the largest amount of discrepancies from all cell types used. It has been demonstrated in multiple studies that primary microglia cannot be cultured in isolation as they de-differentiate soon after that. However, a recent report has suggested that this de-differentiation can occur within hours of isolation (Bohlen et al. 2017). This could provide a possible explanation for the surprising discrepancy in cytokine release between cell lines and primary microglia which had to be purified from a mixed culture to perform the measurements. Cytokine signatures in microglia are dynamic and can rapidly change due to a change in environment which means that the cytokine expression pattern of iron-fed microglia could have been altered by the de-differentiation processes induced by their isolation.

The differences in the effects of iron between the different cell lines and the primary microglia could be due to intrinsic differences in the biology of the different cells. The two cell lines have been shown by the manufacturers and us to retain many microglial characteristics, however they are unlikely to retain all of them considering that they underwent an immortalisation process. Additionally, the discrepancies seen between the primary microglial cells and the mouse microglia point to the possibility that different culturing conditions possibly induce different responses to iron in microglia. The C8B4 cells were cultured by themselves while primary microglia were grown in mixed glia culture that was treated with iron for prolonged periods. It is possible that altered intercellular communication in the mixed culture was responsible for some of the unexpected responses in primary microglia in terms of their cytokine profile.

5.3 Validity of the Microglial Models

5.3.1 Validity of iron-fed microglia as a model for aged microglia

The microglial model of aging presented in this thesis replicates many qualities of aged microglia. Firstly, it was demonstrated that microglia can accumulate iron similarly to how they do in aging. Additionally, they were shown to exhibit impaired autophagy. They also displayed similar changes in the expression of proteins related to aging such as SIRT1 and KV1.3. Their secretory phenotype was also similar to aged microglia – reduced glutamate release, increased reactive oxygen species and increased inflammatory cytokines. Additionally, the successful replication of the role of microglia in major aspects of age-related neurodegenerative disease such as α-synuclein and β-amyloid deposition suggested that this model is a valid representation of aged microglia.

However, despite the many similarities of iron-fed microglia to real aged microglia some important differences should also be noted. Data on microglial numbers in the aged brain points to an overall maintenance of microglial density maintained through proliferation and apoptosis (Askew et al. 2017). In contrast to this data iron-fed C8B4 cells exhibited lower proliferation rates than controls. On the other hand, they also exhibited lower death rates which did result in a similar situation to that observed in the brain. Due to the immortalised nature of the C8B4 cell line it is difficult to make any conclusions on whether this difference could be used to explain any other differences observed between iron-fed and aged microglia.

The cytokine profile of iron-fed microglia compared to real aged microglia also exhibited some discrepancies. In aged mice overall microglia have been found to show an increased expression of both proinflammatory (IL-6, IL-1β, TNF-α) and anti-inflammatory cytokines (IL-10) (Mosher and Wyss-Coray 2014; Norden and Godbout 2013). Aged microglia in the mouse brain have been found to produce more IL-6 while iron-fed primary mouse microglia produced less (Ye and Johnson 1999). They have also been found to produce more IL-10 which was also seen in primary mouse microglia but not in C8B4 mouse microglia. IL-10 was found to be elevated in aged mouse brains stimulated by LPS too. Aged mouse microglia exhibit increased levels of IL-1β while iron-fed mouse primary microglia secreted less IL-1β. On the other hand, TNF-α, found to be increased in aged mouse microglia and similarly in iron-fed C8B4 and primary microglia was unchanged in human microglia.

Changes in cytokine expression have been better studied in aged mouse microglia and less well in humans. The cytokine profile of microglia in the aged human brain in one gene expression study shows decreased levels of IL-10, IL-1β and unchanged TNFα (Olah et al. 2018). On the other hand, another older study shows an increase of IL-1β positive microglia in the aging brain (Sheng et al. 1998). Iron-fed microglia from the human cell line however showed discrepancies in the levels of some of the cytokines mentioned above.

These discrepancies point to the fact that the iron-fed microglia model, while promising is still in its initial stages of development and would need further refining and characterisation in order to understand if it is capable of fully replicating the complex and often conflicting phenotype of aging microglia.

As it has been recently argued, due to the difficulty and costs of culturing real aged microglia and the unsuitability of *ex vivo* microglial harvesting for many types of studies it is still vital that more efficient and practical aged microglial models such as iron-fed microglia are developed and optimised (Koellhoffer et al. 2017).

5.3.2 Validity of other models of microglial aging

The Ercc1$^{\Delta/-}$ mouse model exhibits accelerated aging due to the deletion of the DNA excision repair protein ERCC. That results in genomic instability, one of the hallmarks of aging and a root cause for cellular senescence. Ercc1$^{\Delta/-}$ mouse microglia have been shown to have some characteristics similar to aging (Raj et al. 2014). They exhibited a primed phenotype which has been linked to microglial aging. They were shown to have a larger size and process length compared to control microglia. Their proliferation was also significantly increased. The levels of cytokines such as IL-1β. TNF-α and IL-6 were unchanged until the microglia were stimulated with LPS, when they reacted more strongly than control replicating the actions of primed microglia found in aged mice (Godbout et al. 2005). However they did not replicate the basal increase in cytokine production seen in aged mice (Sierra et al. 2007). They were also found to have increased phagocytosis and increased ROS production. On the other hand aged mouse microglia have been observed to phagocytose less material (Njie et al. 2012) Their transcriptional profile showed changes similar to those in aged microglia such as increase in interferon and chemokine signalling and antigen presentation. Interestingly, they further showed increased EIF2 signalling similarly to iron-fed human microglia(Hickman et al. 2013).

The mouse model of accelerated aging through telomere shortening models aging in a similar way to ERCC deletion (Raj et al. 2015). Telomere shortening causes genomic instability and potentially triggers cellular senescence. also shows microglia that exhibit some aging characteristics. They display a phenotype of increased inflammatory response to LPS but similarly to the Ercc1$^{\Delta/-}$ mouse they did not show any changes in inflammation under physiological conditions. Additionally, they did not show a gene expression pattern of age-associated microglial priming making them an unlikely candidate for an aging microglia model.

The *in vitro* aging mouse microglia model has suggested that microglia convert to an aged phenotype with time in culture (Caldeira et al. 2014). The mechanism for this phenomenon has not been explained but is likely to be a form of replicative senescence. Changes in purified microglia grown *in vitro* for 16 days replicate many age-related changes when compared to freshly purified microglia. They showed reduced autophagy, reduced motility and reduced phagocytosis and increased expression of markers of senescence like SA-β-GAL activity and miR-146a. In a second study they were also shown to be less able to be activated by β-amyloid (Caldeira et al. 2017). However, they displayed characteristics of reduced reactivity such as decreased NFκB signalling and reduced cytokine release which are uncharacteristic of aging microglia (Sierra et al. 2007).

Another *in vitro* model of aging microglia was induced by treating rat microglia in culture with the molecule dexamethasone (Park et al. 2018). Dexamethasone is supposed to mimic microglial exposure to steroid hormones in times of stress and was intended to model microglial behaviour in depression. However, the microglial phenotype that was induced displayed markers of senescence and reduced neuroprotection which were similar to changes as seen in the extended time in culture model such as reduction of cytokine release and phagocytosis and increased SA-β-GAL activity. The morphology of the phenotype they induced was ramified similar to resting microglia. This fact and their reduced cytokine release challenge this model's validity in aging microglia research as mentioned in the study.

5.3.3 Validity of LPS activation studies in microglia

Models of microglial states are used in situations other than aging. Microglial activation is a major area of study because of the role activated microglia play in many diseases that affect the brain. Microglial activation is most commonly modelled by stimulation with bacterial lipopolysaccharide (LPS). LPS can be injected into animals or directly administered to cell culture. It results in a strong inflammatory response which is often used to study microglial response to neurodegeneration or protein aggregates in AD and PD (Nazem et al. 2015) . LPS injection has been used to induce microglial activation and neurotoxicity in the substantia nigra of rats(Subramaniam and Federoff 2017). In AD models, LPS has been found to induce microglia to phagocytose more β-amyloid in some studies (Mandrekar-Colucci and Landreth 2010; Herber et al. 2007; Liu et al. 2005; DiCarlo et al. 2001). On the other hand LPS treatment has been shown to increase β-amyloid deposition in a mouse model of AD (Sheng et al. 2003). Some of the heterogeneity in these results could possibly be explained by administration methods where different amounts of LPS reach the brain depending on whether in was injected

peripherally or intracerebrally because of blood-brain barrier penetration. Additionally, LPS can produce different effects on microglia depending on the number of times they are exposed to it. Repeated LPS administration of LPS was reported to induce higher SA-β-GAL activity indicating senescence in BV2 microglial cells. Alternatively, in a mouse model of AD one-off exposure to LPS can result in increased inflammation and amyloidosis while multiple exposures result in an anti-inflammatory response which is more neuroprotective termed as tolerance (Wendeln et al. 2018). Similar results have been seen in another study on repeated LPS injection in mouse models of acute brain injury (Chen et al. 2012). What this study suggests is that microglia have a complex response to LPS that can change over time and in different models. This finding is not surprising considering that microglia are highly heterogeneous cells but is indicative of the unsuitability of LPS for aging microglia studies.

5.3.4 Validity of quantitating morphological changes in microglia

Microglia are naturally highly reactive cells and are capable of changing their morphology in response to any external circumstances. Morphological changes in microglia have been observed with microscopy for a long time and are interesting visual cue for the changing phenotype of a cell. Newer methods have been described that allow 3D modelling and tracking of live movement and large-scale automated quantitation of microscopy data (Verdonk et al. 2016; Heindl et al. 2018; Ljosa and Carpenter 2009; Weinhard et al. 2018). Additionally, live imaging techniques allow for microglia to be monitored without fixing which can alter morphology and can be used on microglia behavioural studies. (Dailey et al. 2013). However, quantifying morphological changes doesn't present much value in terms of characterising the molecular phenotype of these cells. Microscopy data is normally not truly quantitative as getting an unbiased sample with manual measurements is very difficult. Morphological changes is an abstract quality that historically has not been not as much measured as it was judged by the person analysing the data (Hamilton 2009). Only recently have more quantitative methods been described that attempt to give a more unbiased measurement of microglial morphology (Davis et al. 2017) However, the morphological changes in microglia are driven by changes in the molecular environment of the cell, changes in transcription, levels and activity of proteins, signalling molecules and metabolites – all molecules that can be measured more precisely with biochemical assays. Thus, even though morphological changes are interesting to observe, the real value in studying changes in microglia comes from measuring changes on the molecular level.

5.3.5 Relevance of general activation state studies.

As explained in the Introduction, microglia have been shown to have a spectrum of activation states that are difficult to put into specific categories. Studying activation states of microglia is therefore important for the purpose of understanding their role in the brain more fully. This heterogeneity means that microglia will react differently to damage or infection depending on where they are located in the brain and what type the insult. Many studies have attempted to track microglial activation in different models of neuronal injury or disease (Thiel and Heiss 2011; Bosco et al. 2011; Morrison and Filosa 2013; Deng et

al. 2009; Mac Nair et al. 2016; Wohl et al. 2010). Methods for identifying and classifying activation states in microglia are currently being developed. Those are largely morphology based and are particularly relevant in *in vivo* studies (Megjhani et al. 2015; Hovens et al. 2014; Eggen et al. 2013; Verdonk et al. 2016; Zanier et al. 2015; Davis et al. 2017).

However, it has proven difficult to define specific activation states that microglia can fall into such as the likes of M1 and M2 for macrophages(Boche et al. 2013; Ransohoff 2016).The evidence of microglial priming in the aging brain suggests that aged microglia would react differently to damage or insult to young microglia(Dilger and Johnson 2008).Examples of cases where the activation of aged microglia has been studied are peripheral LPS injection in aged mice and peripheral E.coli injection in aged rats (Godbout et al. 2005; Wynne et al. 2010; Barrientos et al. 2009; Barrientos et al. 2006). In both cases aged microglia were shown to release increased levels of proinflammatory cytokines. In other studies aged microglia have been observed to show reduced migration, phagocytosis and recruitment of immune cells when activated by damage in murine models(Norden and Godbout 2013). A potential future avenue for characterisation of the iron-fed microglia aging model would be to study it in the context of murine models and assess whether iron-fed microglia react similarly to aged microglia in this context in terms of their cytokine release and other markers of activation.

5.3.6 Senescence and SASP in aged and iron-fed microglia

Cellular senescence has been shown to occur in many replication competent cell types in the brain and is thought to be a major contributor to inflammation in the brain in old age (Chinta et al. 2014). It is characterised by arrested growth due to elevated DNA damage and oxidative stress that results in increased SA-β-GAL activity, $p16^{INK4a}$ expression and the SASP among others. Interestingly, cells *in vitro* have been found to also naturally accumulate iron as a characteristic of the process of acquiring senescence (Masaldan et al. 2018; Killilea et al. 2003). SASP is an integral part of the characteristics of a senescent cell. It includes many signalling molecules such as proinflammatory cytokines, chemokines, reactive oxygen species, growth factors and proteases that can cause a significant negative impact on surrounding cells and is linked to the aging and ND brain (Chinta et al. 2014).

Aged microglia have been shown to display many characteristics of senescence including iron accumulation and SASP-congruent increased secretion of cytokines and ROS. As a persistent population of cells that maintain their numbers through division they are at high risk for senescence. However, identifying and purifying senescent microglial cells *in vivo* in order to characterise their specific SASP has not been achieved yet. In chronic senescence which would be the type present in brain microglia, SASP can be more heterogenous than in cases of *in vitro* replicative senescence (van Deursen 2014). Nevertheless, understanding better how microglia undergo senescence by characterising their specific senescent phenotype and SASP is very important for determining how they affect the molecular environment of the aging brain, especially in terms of inflammation.

Iron-fed microglia also display many signs of cellular senescence. Those include iron accumulation, mTOR activation, NFκB activation, increased proinflammatory cytokine release (IL-1β, IL-6, IL-8, KC-GRO) and ROS secretion. Additionally, they showed lower

rates of proliferation in the C8B4 cell line whose proliferation was not artificially manipulated. These similarities position iron-fed microglia as a useful model for characterising the SASP that aged microglia can have. Further investigation into how well iron-fed microglia replicate other markers of senescence would be essential if they are to be applied as a model of senescent cells.

5.4 Future work

5.4.1 Further characterisation of the iron-fed microglial phenotype.

The changes that microglia undergo when treated with iron resemble those of aged cells. In order to comprehensively study the phenotypic changes that iron-fed microglia undergo it would be useful to have a complete dataset of their transcriptional phenotype. Performing RNAseq on the iron-fed SV40 human and C8B4 microglial cell lines and also on primary mouse microglia and comparing those to untreated controls and existing datasets for aged and diseased human and mouse microglia would help to identify major pathways that are affected in iron-fed microglia and how those compare to the changes seen in aged or diseased microglia. That would allow us to identify molecules and pathways to target in order to understand how the senescent phenotype can be replicated by iron accumulation. It will also allow us to study how iron-fed microglia resemble or differ from microglia in neurodegenerative disease. Additionally, this will make it possible to screen aged tissue samples for markers of microglial dystrophy as very few of those have been identified to date. Other senescence markers identified in the literature such as SA-β-GAL activity, $p16^{INK4a}$, CD200 and CX3CR1 expression and activity could also be investigated. Measuring changes in activity in iron-fed microglia is also very important for fully characterising the model. Microglial motility and phagocytic ability could be measured in the cell culture models to identify if those are reduced as seen in aged microglia. An ATP-triggered motility assay and a latex bead or β-amyloid phagocytosis assay could be employed to assess those effects. Phagocyting status of microglia could also be measured by Galectin-3 staining (Shobin et al. 2017). Additionally, even though the cytokine profile of iron-fed microglia has been characterised, TGF-β – an important anti-inflammatory cytokine found to be altered in aged microglia in some studies is yet to be measured. That could be achieved through utilising an immunoassay in a similar manner as other cytokines in the conditioned medium were measured. Finally, decreased expression of SIRT1 has been seen in iron-fed microglia. A possible outcome of less SIRT1 activity can be increased levels of acetylated FOXO3a which can lead to increased levels of oxidative stress in microglia. Measuring SIRT1 activity and levels of FOXO3a acetylation would allow for assessing if the FOXO3a pathway is also involved in the iron-fed microglia phenotype.

Gathering this information would make is possible to assess how iron supplementation pushes microglia into the aged phenotype. This may be due to membrane oxidation and DNA damage which can be measured with standard biochemical techniques. In order to fully understand this mechanism phenotype reversal will also have to be attempted.

5.4.2 *In vivo* application of iron-fed microglia

Another important avenue to investigate would be the application of the aged microglia model *in vivo*. Therefore, it is very important to determine if iron-fed microglia transplanted into mouse brains will induce phenotypic changes in neuronal cells similar to those seen in the culture models. For this to be achieved control and iron-fed microglia could be transplanted in to live rodent brains. This has been previously attempted with both primary and cell line microglia (Leovsky et al. 2015; Narantuya et al. 2010; Watanabe et al. 2002; Takata et al. 2007). The areas where transplantation would be attempted would ideally be areas of the brain that are affected by aging such as the substantia nigra. The microglia would be transplanted using stereotaxic injection. The first hurdle to overcome would be to confirm microglial survival. This can be done by labelling the microglial cells with a long-term fluorescent marker such as carboxyfluorescein succinimidyl ester. In the initial stages post transplantation mice would be sacrificed and brain sections probed for the fluorescent marker and for microglia with high levels of ferritin expression. An important issue to be considered is that iron-fed microglia may have a lower rate of survival compared to healthy controls which may affect the results of further tests. After confirming the survival of the microglia in mouse brains the mice would be monitored for changes in behaviour such as decreased locomotion and general ill health. Tests such as rotarod and forepaw stride length could be utilised. The effect of iron-fed microglia on neuronal viability and signs of neurodegenerative disease could be measured. Standard methods such as TUNEL staining could be used to assess neuronal viability. α-synuclein and β-amyloid levels together with signs of aggregation and changes in markers of dopamine metabolism could also be measured to assess the effect of iron-fed microglia on markers of neurodegenerative disease. Additionally, microglia could be monitored for signs of dystrophy *in situ* which would be a much more appropriate environment to investigate such changes compared to *in vitro*. If changes related to neurodegeneration can be replicated it would be important to attempt microglial phenotype reversal *in vivo* to find out if that can prevent downstream pathological changes in the CNS.

5.4.3 Phenotype reversal

Understanding what produces the phenotype caused by iron supplementation can be greatly aided by attempting to reverse it. The effects could be caused by the elevated levels of iron themselves or by lasting molecular damage to the microglia caused by the iron accumulation. One avenue of investigation is applying iron chelators to the iron-fed microglia to find out if lowering the levels of iron reverses, the senescent phenotype. Common iron-chelators that can cross the blood-brain barrier are deferiprone and deferoxamine. Deferiprone has been shown to improve the pathology of neurodegeneration with brain iron accumulation (Abbruzzese et al. 2011). Deferoxamine is used to treat iron accumulation following brain haemorrhage (He et al. 2016; Hua et al. 2008).Those can be applied to the cell culture model and *in vivo* models to reduce iron levels. That can be estimated through measuring iron storage, ferritin levels and iron staining. Improvements in migration, phagocytosis, cytokine release, autophagy and ER stress would suggest that the phenotype has been reversed. If that is the case, other iron

chelators can be investigated. A potential avenue of research can be phytochemicals such as polyphenols and flavonoids are molecules contained in plants with reported beneficial effects on neurodegeneration. They are a type of natural iron chelators and have been shown to be very effective in cell culture (Reznichenko et al. 2006; Hatcher et al. 2009; Ferlazzo et al. 2016). Epigallocatechin gallate (EGCG) is a polyphenol contained in green tea and has been proposed as a potential therapeutic for neurodegeneration as it has shown neuroprotective and antioxidant effects *in vitro*. EGCG has also been found to chelate iron but its ability to cross the blood-brain barrier has been shown to be quite low thus limiting its potential effectiveness *in vivo* (Singh et al. 2016; Pervin et al. 2017). Quercetin, a flavonoid shown to chelate iron similarly has low blood brain barrier permeability but that could potentially be enhanced by co-administration of α-tocopherol (Ferri et al. 2015).

A possible outcome of iron depletion would be that the damage caused by iron is irreversible. In that case it would be important to pinpoint which changes in the cellular metabolism are mechanistic to the senescent phenotype. If for example iron caused DNA damage removing iron from the system would not reverse that. However, by stimulating DNA repair mechanisms the phenotype could possibly be reversed suggesting that DNA damage is the underlying mechanism of the senescent phenotype. When looking into reversing iron-mediated damage it is important to consider the increased division rates of cell lines. That presents a limited window of opportunity for reversing damage as proliferation will obscure any measurable changes. A slower proliferating cell line like C8B4 would be more suitable for these experiments than fast proliferating ones like BV2 or the SV40 human microglia cell line. Even then, attempting this type of phenotype reversal would be best in primary microglia or *in vivo*.

Another potential strategy to reverse the aged microglial phenotype is exposing them to young microglia. In a mouse brain slice culture model, it was observed that treating aged microglia with the conditioned medium of young microglia restored their viability and ability to phagocytose β-amyloid (Daria et al. 2017). That was also replicated by treating the aged cells with granulocyte-macrophage colony-stimulating factor (GM-CSF). Treating iron-fed microglia with conditioned medium from young microglia may result in a reversal of the aged phenotype in a similar way. GM-CSF is known to drive microglial proliferation which was necessary for the enhanced amyloid clearance in that model. It is possible that in our model more factors secreted by young microglia such as cytokines may play a role in reversing all the age-related changes observed.

Resveratrol treatment has been shown to reverse cellular senescence *in vitro* and to have a direct action on reducing the release of SASP cytokines, activating SIRT1 and modulating levels of mRNA splicing factors (Fuggetta et al. 2016; Sinclair and Guarente 2014; Markus et al. 2011; Matos et al. 2017; Latorre et al. 2017). Treating iron-fed microglia with resveratrol could result in a recovery in SIRT1 levels and a reduction in inflammatory cytokine levels and possibly the amelioration of other markers of the aged phenotype. Different molecular analogues of resveratrol have also been developed that have an action on a smaller subset of resveratrol targets (Latorre et al. 2017). If resveratrol is effective in reversing the phenotype, testing which of those analogues if capable of doing the same would allow to trace back which particular pathway causes the iron-fed microglia phenotype.

Finally, reversing the phenotype of iron-fed microglia could potentially be achieved by applying the drug metformin that has in a number of studies been shown to extend human lifespan through a number of potential mechanisms that have been linked to nutrient sensing including decreased IGF-1 signalling, inhibition of mTOR, reducing ROS and DNA damage and lowering inflammation. As it is very likely that these pathways are involved in the generation of the iron-fed aged microglial phenotype it is possible that metformin could be used to improve their viability. Additionally, metformin has recently been applied to induce alternative activation in microglia and promote microglia assisted healing in different conditions which suggests that it has a beneficial effect on pathological conditions in the brain (Inyang et al. 2016; Ge et al. 2018; Jin et al. 2014). Metformin's long-standing usage as a drug in humans and the numerous studies done on animal models make it an interesting potential candidate for microglial aged phenotype reversal for *in vivo* applications.

Being able to reverse the aged phenotype would be of benefit not only to the aged microglia cell culture model but also to its *in vivo* application and potentially for developing treatments for age-related neurodegenerative disease.

5.4.4 Role of microglia in other neurodegenerative diseases with an iron accumulation component

Iron homeostasis in the brain is finely tuned and the iron content of different parts of the brain is heterogenous. The aging process can lead to the accumulation of iron in certain cell types and brain regions. Iron has been found to be naturally stored in oligodendrocytes, but it accumulates with age in microglia and astrocytes. An increase in iron-positive microglia and astrocytes have been found in the hippocampus, cortex, basal ganglia and the cerebellum. Iron has also been found to accumulate as ferritin in specific areas of the brain such as the globus pallidus, substantia nigra and the cortex. Additionally, increases of iron as neuromelanin have been found in catecholaminergic neurons in the substantia nigra. The reason behind this accumulation has not been fully elucidated. A possible explanation is increased vascularization of the brain. Alternatively, a leaky blood-brain barrier could result in the penetration of excess iron into the brain. Increased inflammation could also be the underlying reason for the increase in brain iron (Angelova and Brown 2015; Ward et al. 2014). Whatever the cause for the increases in brain iron it is important to point out that the regions where it accumulates with age are often affected by ND as well.

Iron can have a multitude of negative effects in an organism. In fact, there are some diseases in which accumulation of iron or dysregulation of iron metabolism could be a central disease driving mechanism.

Neurodegeneration with brain iron accumulation (NBIA) is a cluster of diseases that are characterised by intellectual disability, movement impairment and abnormal deposition of iron in the basal ganglia. There are ten subtypes of NBIA currently identified (pantothenate kinase-associated neurodegeneration (PKAN); phospholipase A2-

associated neurodegeneration (PLAN); mitochondrial membrane protein-associated neurodegeneration (MPAN); and beta-propeller protein-associated neurodegeneration (BPAN), fatty acid hydroxylase-associated neurodegeneration (FAHN), coenzyme A synthase protein-associated neurodegeneration (CoPAN), neuroferritinopathy (NF), Kufor-Rakeb syndrome (KRS) and aceruloplasminemia (ACP)) where PKAN is the most common (Gregory and Hayflick 2011). All these heritable diseases are caused by a mutation in a single gene and are considered ultra-rare. An interesting form of NBIA is neuroferritinopathy (NF) as it demonstrates a direct link between dysregulated iron storage and neurodegeneration. It is caused by mutations in the gene *FTL1* coding for the ferritin light chain protein (Chinnery 2010; Kumar et al. 2016; Levi and Rovida 2015). This leads to low serum ferritin levels but accumulation of iron and ferritin inclusions both in the basal ganglia in a pattern similar to normal aging and in other tissues in the body. The disease manifests usually late in adulthood as progressive chorea and dystonia and many other motor symptoms and also cognitive decline. The iron and ferritin deposits are found both extracellularly and in various cell types in the brain including astrocytes, oligodendrocytes, cerebellar granule cells, Purkinje cells and microglia. The proposed molecular mechanism for the disease is that the mutations that are linked to NF seem to reduce the ability of ferritin to store iron. The increased free iron can then cause oxidative damage to which the brain is particularly sensitive thus explaining why it's only the nervous system that is affected. Even though iron accumulation is seen in microglia in NF their role in the disease has not been studied.

Another disease directly linked to iron is Friedrich's ataxia (FA). FA is a hereditary neurodegenerative disease and is the most common of all hereditary ataxias. It usually develops in childhood and leads to the degeneration of the spinal cord and peripheral nerves and also the cerebellum leading to motor symptoms but cognitive ability is not impaired in FA (Bürk 2017). Other commonly found symptoms include heart problems and diabetes. FA is a serious condition and often results in a severely reduced lifespan. It is caused by a mutation in the gene coding for the protein frataxin. The mutation causing FA is an expansion of a trinucleotide repeat in the first intron of the frataxin gene leading to reduced expression of the protein (Bürk 2017). Frataxin is a mitochondrial protein that is thought to act as an allosteric modulator of iron-sulphur-cluster protein assembly. It has an essential role in cellular function as frataxin KO in mice is lethal. Frataxin deficiency results in mitochondrial iron accumulation and cytosolic iron depletion. It also results in respiratory chain disruption which elevates the amounts of H2O2 in mitochondria that oxidise the accumulated iron and thus resulting in further oxidative stress through the Fenton reaction. The potential role of microglia in FA has not been extensively studied but increased ferritin staining in microglia has been found in the dentate nucleus of FA patients. Additionally frataxin deficiency in the microglial cells of a FA mouse model has been shown to induce DNA damage and increase microglial inflammatory response (Shen et al. 2016). Treatment of FA patients with the iron chelator deferiprone has resulted in reduced iron accumulation in the cerebellar dentate nucleus and an improvement in motor and speech symptoms further confirming the central role iron accumulation plays in this condition (Bürk 2017; Koeppen et al. 2007).

From these examples it is clear that iron plays an important role in the brain and that iron dyshomeostasis is implicated in some way in many ND and can potentially cause a lot of damage in the nervous system. This topic has been also reviewed in Angelova and Brown

2015 (Appendix 7.1). Therefore, the accumulation of iron in microglia seen with aging is unlikely to be without negative consequences as also demonstrated in the iron-fed microglial model.

5.5 Applying iron-fed microglia to the study of ß-Amyloid

5.5.1 Relevance

The main finding of chapter 2 that reduced IDE secretion by microglia results in reduced β-amyloid degradation has been observed in literature. Reduced IDE levels have been measured in AD brains and mouse models of AD. However, in AD, the levels of β-amyloid are not only maintained by levels of other proteases but also by microglial phagocytosis, a process that was not investigated in this study.

5.5.2 Advantages and disadvantages of using the iron-fed microglia model in the study of ß-amyloid

Using iron-fed microglia to model microglia in AD has its benefits and drawbacks. The human microglial cell line that was used in chapter 2 shows many changes similar to microglia in AD.
The morphological changes that were seen in the microglial cell line have been identified in the microglia of human AD brains, namely reduced process length and branching (Davies et al. 2017). The increased ferritin expression in the aging microglia model has also been seen in AD. Ferritin positive microglia have been identified in human AD brains in an even higher proportion than normal aging (Lopes et al. 2008). Additionally, increases in ferritin bound iron have been linked to earlier age of onset of AD in men (Bartzokis et al. 2004). However, both of these studies only show a tangential link between increased ferritin expression and the microglial phenotype in AD. Decreased autophagy in microglia is a marker of aging that not been heavily researched in AD. Primary microglia from human AD brains show reduced expression of beclin 1, an early stage driver of autophagy. Additionally, inhibiting key activators of autophagy in microglia has been shown to reduce β-amyloid clearance and also to increase the release of proinflammatory factors (Lucin et al. 2013; Cho et al. 2014). Also, ER stress has been implicated in AD and is thought to be one process through which neurons can die, while microglia have not yet been observed to exhibit more of it in AD (Salminen et al. 2009).
The expression of protein markers of microglial aging has also been validated by studies on microglia in AD. KV1.3 expression, perhaps unsurprisingly is another factor where the aged microglia model replicated changes seen in AD, as KV1.3 positive microglia are present in both human and rodent models of AD (Maezawa et al. 2018; Rangaraju et al. 2015). The SIRT1 deficiency observed in the aging microglia model replicated changes seen in aging microglia. Microglial SIRT1 decline has been linked to increased IL-1β production as seen in microglia in AD (Cho et al. 2015). Additionally, overall decline in SIRT1 has been correlated with AD pathology (Julien et al. 2009). Even though the data available points in this direction, there is no data on the actual levels of SIRT1 in microglia in AD.

All these points suggest that the iron-fed aged microglia model replicates the behaviour and markers of microglia in AD well and is therefore a viable tool for incorporating microglia into AD studies.

5.5.3 Future Work

The production of β-amyloid was also affected by microglial conditioned medium. As α- and β-secretases expression was already investigated the data suggest that the observed increase in β-amyloid could be due to an increase in γ-secretase activity, confirmed by the results from the Notch cleavage assay. More γ-secretase activity could be caused by an increase in the expression of the components of this protease. The expression of PS1, a main component of γ-secretase has been shown to be induced by impairment in autophagy (Ohta et al. 2010). Measuring PS1 levels and then assessing neuronal cells treated with microglial conditioned medium for autophagy impairment would be a potential strategy to explain another avenue through which β-amyloid levels could increase. Another avenue through which this work could be taken further would be to investigate if iron-fed microglia are capable of phagocytosing β-amyloid. In AD β-amyloid phagocytosis by microglia is reduced. This could be investigated through an *in vitro* assay on fluorescently tagged β-amyloid internalisation as described previously (Lian et al. 2016). Another question that is yet to be investigated in the context of the effect of iron-fed microglia on β-amyloid deposition is whether iron-fed microglia affect β-amyloid oligomerisation. It has already been shown that protofibril forms of β-amyloid are more readily internalised by microglia than monomers and lead to greater levels of activation(Gouwens et al. 2016). Oligomers and protofibrils also seem to lead to differential modes of activation in microglia (Sondag et al. 2009). However, whether factors released by microglia affect oligomerisation rates of β-amyloid is not known. The oligomerisation process is thought to occur intracellularly in neurons but also extracellularly and is possibly driven by β-amyloid fibrils (Walsh et al. 2000; Cohen et al. 2015). Therefore applying conditioned medium from iron-fed microglia to neuronal cultures would allow for any changes in β-amyloid oligomer levels to be measured using molecular probes or antibodies (Lee et al. 2017).

5.6 Applying iron-fed microglia to the study of α-Synuclein

5.6.1 Relevance

α-synuclein levels have been shown to be elevated in areas of the brain affected by PD in multiple studies. The finding presented in Chapter 3 provides one possible explanation for how this can occur. The role of microglia in PD pathology has been generally recognised as many studies have described how α-synuclein can induce microglial activation. TNF-α levels have been shown to be increased in PD and Microglia positive for TNF-α have been identified in the substantia nigra in PD. Additionally, the toxicity of TNF-α produced by microglia on dopaminergic neurons has been observed in many studies (Lull and Block 2010). However, the finding that TNF-α released by aging microglia can increase the expression of α-synuclein has had very limited coverage in literature as mentioned in the discussion of chapter 3. A cell culture study investigating TNF-α released by activated

microglia also found an increase in α-synuclein levels but explained them through reduced degradation by reduced lysosome acidification induced by TNF-α. They observed no change in mRNA levels for α-synuclein in contrast to results reported in this thesis that suggested elevated α-synuclein transcription. That could be due to differences between the cell lines used in the two studies. However, disrupted autophagic flux is another potential pathway that could also have affected the α-synuclein levels in SHSY5Y cells treated with microglial conditioned medium(M.-X. Wang et al. 2015).
The main finding of chapter 4 that increased levels of α-synuclein increase the toxicity of α-synuclein oligomers through elevated oxidative stress is novel but is generally supported by the findings of many other studies. As mentioned previously elevated levels of wild type α-synuclein are causative of a form of parkinsonism so therefore must contribute to toxicity (Olgiati et al. 2015). Additionally, elevated oxidative stress has been recognised as a disease driving process in PD with significant evidence for oxidative damage in various cellular components and as already discussed PD patients have been found to have elevated Fe^{2+}(X. Chen et al. 2012). Fe^{2+} as a source of ROS has also been identified in PD with some dopaminergic neurons also presenting increased iron levels in the substantia nigra. Interaction and subsequent reduction of iron by dopamine or other ferrireductases have been proposed to be the source of Fe^{2+} in PD (Ponting 2001; Hare and Double 2016).Therefore the finding that elevation of α-synuclein – another ferrireductase could lead to the same elevation in Fe^{2+} is not surprising. Additionally, increased levels of α-synuclein have been shown to increase the toxicity of other exogenous factors such as copper, suggesting that appropriate α-synuclein levels are important for cellular homeostasis (Anandhan et al. 2015). As discussed in chapter 4 the balance in expression between α- and β-synuclein also seems to be implicated in α-synuclein toxicity. These results point to another potential avenue for how PD pathology can proceed through aged microglia causing elevated α-synuclein, disrupting α and β-synuclein homeostasis and driving neurotoxicity through elevated oxidative stress as a result.

In conclusion, the data presented in this thesis provide a valid pathway on how aged microglia can influence PD pathology from a previously unexamined angle that fits with the current knowledge on how PD proceeds in humans (Imamura et al. 2003; Subramaniam and Federoff 2017).

5.6.2 Advantages and disadvantages of using the iron-fed microglia model in the study of α-synuclein

Using the iron-fed microglial model in the study of α-synuclein in disease has undeniable merits. Microglia have been shown to play a major role in PD or other synucleinopathies. The iron-fed aged microglial model shows changes in their phenotype and behaviours that are similar to changes detected in microglia in PD or other synucleinopathies. Firstly, the increase in ferritin bound iron in iron-fed microglia replicates the increases in ferritin bound iron and ferritin positive microglia in the *substantia nigra* seen in PD (Jellinger et al. 1990). Microglial activation is thought to be a major pathological process in PD. In fact, inactivating microglia ameliorates PD pathology in mouse models treated with MPTP (M. Lu et al. 2016). Microglia in PD have been shown to release higher levels of IL-6, TNFα

and IL-1β similarly to iron-fed C8B4 microglia. Additionally, microglial KV1.3 has been found to be essential for the killing of neurons (Fordyce et al. 2005). On top of that microglia in PD have been found to release increased levels of ROS(Peterson and Flood 2012). On the other hand, the cytokine profile observed in iron-fed primary mouse microglia is not as similar to microglia in PD as the iron-fed C8B4 cell line. They only showed an increase in the secretion of TNF-α suggesting that the iron feeding protocol at its' current iteration doesn't replicate the changes seen in microglia in PD in primary cells as well as it does in cell lines.

5.6.3 Future Work

Future work that can benefit from using model senescent microglia is assessing other ways in which aged microglia could affect α-synuclein deposition. Microglia have been shown to be effective at phagocytosing extracellular α-synuclein. Iron-fed microglia can be utilised to investigate whether aged microglia which have been shown to have impaired phagocytic ability are less capable of clearing extracellular α-synuclein deposits. Internalisation of fluorescently labelled α-synuclein by microglia can be measured by confocal microscopy. Additionally, neuronal co-culture can be used to assess whether levels of α-synuclein phagocytosis are altered.
Another potential mechanism through which iron-fed microglia could increase α-synuclein aggregation is through the release of nitric oxide (NO). Increased release of NO can lead to α-synuclein nitration which has been reported to increase rates of aggregation(Shavali et al. 2006). Additionally, elevated α-synuclein nitration has been observed in the aging primate substantia nigra(McCormack et al. 2012). Measuring NO released in the conditioned medium of iron-fed microglia, applying a NO synthase inhibitor and comparing α-synuclein nitration in neuronal cells will make it possible to assess whether aged microglia can affect α-synuclein deposition in that manner.
The effect of microglia on neuronal FOXO3a activation could also be further investigated. As it was demonstrated that iron-fed microglial conditioned medium resulted in increased α-synuclein expression and increased α-synuclein expression led to increased pro-apoptotic FOXO3a activity it is important to understand the mechanism of activation of FOXO3a more fully. Acetylated FOXO3a has been shown to have proapoptotic activity and can be affected by declining levels of SIRT1. Recent work in our lab performed by Verity Mitchener showed that applying conditioned medium from iron-fed microglia onto SHSY5Y cells resulted in a reduction of SIRT1 expression. Therefore, assessing acetylated FOXO3a levels in cells treated with conditioned medium would provide evidence for another potential pathway for FOXO3a activation in this context.

5.7 Applying iron-fed microglia to the study of aging

5.7.1 Relevance of the iron-fed microglial model to the study of aging

Even though the iron-fed aged microglial model was not applied to the study of brain aging, it could replicate major changes seen in the aging brain the role of aged microglia in which has not been well studied. Brain aging even in non-pathological conditions is characterised by diminished cognitive ability that could be due to impaired synaptic

plasticity. Maintenance of synaptic plasticity has recently been recognised as an important role for microglia both in development but also in the adult organism (Wu et al. 2015). Iron-fed microglia as a model for aging microglia could be used to investigate the role of microglia in these processes. A well-studied form of synaptic plasticity is long-term potentiation. Appropriate levels of reactive oxygen species have been shown to play an important role in long-term potentiation in the hippocampus (Serrano and Klann 2004). Both aged and iron-fed microglia show dysregulated release of ROS which could affect these processes. Additionally, overproduction of IL-1β by microglia has also been linked to impaired long-term potentiation in the hippocampus (Patterson 2015). IL-1β levels are increased in iron-fed microglia providing another potential avenue through which brain aging could be replicated by the aged microglia model.

Genomic instability is another hallmark of aging that aged microglia could play a role in. It has been shown that chronic inflammation can lead to genomic instability in studies in the context of cancer (Lin et al. 2016; Yan et al. 2009). The fact that aged microglia drive increased inflammation in the aging nervous system suggests that they also play a role in the genomic instability seen in aging neurons (Burhans and Weinberger 2007). Lower levels of SIRT1 may be a key player in these events (Oberdoerffer et al. 2008) and conditioned medium from iron-fed microglia was shown to reduce SIRT1 levels in neuronal cells. This suggests that iron-fed microglia could potentially induce genomic instability in neurons thus expanding the role that aged microglia could play in inducing overall brain aging.

5.7.2 Future Work

This model can be used to further the study of brain aging. Iron-fed microglia that show a dysregulated release of ROS and increased IL-β production can be used to model the role that aged microglia could play in long-term potentiation. That can be achieved by using the conditioned medium model in cultured neurons and measuring but can also be applied to *in vivo* transplantation of iron-fed microglia and testing for changes in memory or behaviour. If long-term potentiation is affected by microglial conditioned medium the molecule or molecular responsible for those changes could be identified. The effect of microglia in neuronal SIRT1 expression could also be pursued further by measuring genomic instability that could result as reduced levels of SIRT1. Additionally, identifying any particular components of iron-fed microglia conditioned medium that triggered the change would also be beneficial.

5.7.3 Likelihood of iron-fed microglia being used as a model of aged microglia and potential drawbacks

The main advantages of this model are its simplicity and ease of use. It can be induced in multiple types of cells and can be maintained for long periods. Additionally, using microglial cell lines allows for generating large amounts of cells for studies. That theoretically makes the model easily applicable to many types of studies and thus likely to be used in the future. However, at this point in time the iron-fed microglia aging model has

drawbacks. The treatment with iron seems to induce a slightly different phenotype in different cell lines and in primary microglia. That may be due to the specific biological characteristics of the cell lines used or due to the natural variation in microglial phenotypes. Comprehensively characterising the effects on iron on microglia on the transcriptional and protein expression level in multiple versions of the model is an important step in applying it to aging research. Additionally, the lack of data on the genotype of dystrophic microglia in the brain makes it difficult to compare iron-fed microglia model to them.
Another potential drawback of using cell lines in this model is the low proliferation rates and long lifespan of human microglia. Those characteristics have been linked to hallmarks of aging such as stem cell exhaustion and telomere shortening and also in potential generation of very heterogenous populations of microglia in the aging brain parenchyma (Mosher and Wyss-Coray 2014). Rapidly dividing genetically homogenous cell lines may have reacted differently to iron accumulation than microglia in the brain would have over the decades of their lifetime.
On the other hand, using primary microglia to generate the model has its own unique set of drawbacks as maintaining microglial phenotype *ex vivo* is more technically challenging and can be influenced by the culturing methods applied to the cells. It has been shown that primary microglial cells undergo significant gene expression changes when acutely isolated and that can occur very rapidly. This suggests that isolated primary microglia also don't fully match the gene expression profile of microglia *in situ* which can also apply to aged microglia (Bohlen et al. 2017).

5.7.4 Improving the model

Taking into account the drawbacks of this model it's important to explore whether a better model of aging microglia could be made. One of the most important issues to address would be the discrepancies in the response of different microglial cells to the same treatment with iron. It is possible that due to differences in biology such as division rates different cell lines and primary cells have a different optimal concentration of iron that generates the iron-fed aged phenotype. Optimising that concentration would be central to improving the versatility of the model. Additionally, if data were to become available on the actual concentrations of iron in the microglia of the aged brain the model could be tweaked to reflect those more closely.

Another avenue to look into would be a more direct approach. Understanding which genes drive the changes detected in the iron-fed phenotype and manipulating their activation to replicate the same effects could generate a very effective model. The gap in knowledge about the molecular root of the changes that occur in aged microglia would make that very difficult. It is possible that when it comes to iron-fed microglia the changes that occur as in aging are multifactorial and seemingly cannot converge down to a change in a single pathway. That leaves the iron supplementation method as a much more easily achievable strategy to model aged microglia.

5.7.5 Issues with using neuronal cell lines

Another factor that affects the applicability of iron-fed microglia to the study of aging is their interaction with neurons. The studies described in this thesis have used an immortalised neuronal cell line to study the effect of iron-fed microglia on neurons. However, that made it difficult to measure the model's long-term effects as cell lines are not as differentiated as neurons and also have high division rates. An additional drawback was the difficulty in measuring effects on neuronal viability and synaptic transmission. Therefore, primary neuronal culture would be necessary in order to fully explore these important research questions.

5.8 Other Future Work

The iron-fed aged microglia model has been shown to have utility in the study of ND and in particular in influencing β-amyloid and α-synuclein production and metabolism. It would be beneficial to apply this model to the study of other proteins implicated in common ND.

5.8.1 Tau

Dystrophic microglia have been identified in the vicinity of tau pathology in AD brains (Streit et al. 2009). Microglia have also been found to phagocytose tau and to be activated by the presence of tau aggregates(Morales et al. 2013; Bolós et al. 2015). Microglial neuroinflammation is also thought to promote the hyperphosphorylation of tau and drive tau pathology (Maphis et al. 2015; Cherry et al. 2016). Therefore, the aged microglial model could be used to investigate further the role of these cells in tau deposition. The effect of microglial conditioned medium on tau expression and hyperphosphorylation rates in neuronal cells are potential strategies to investigate how iron-fed microglia could affect this protein. If those are found to be affected the molecules present in conditioned medium that cause these changes could be identified. Those could be cytokines, ROS or other signalling molecules. Another potential process through which microglia could increase tau deposition could be through reduced phagocytosis. Measuring tau phagocytosis by iron-fed microglia would allow to test whether the model can replicate disease conditions and provide another explanation for how microglia drive AD pathology.

5.8.2 Prion protein

The aggregation of the prion protein is a major driving mechanism of prion disease. Activated microglia have been found to co-localise with PrP^{Sc} deposits in the brain of CJD patients. Microglia in the prion diseased brain exhibit a mixed cytokine profile with elevated TNF-α and IL-1β. Additionally, inflammation in CJD has been associated with elevated NFkB pathway activation (Obst et al. 2017). Inflammation driven by microglia is thought to play a major role in prion diseases some of which such as sporadic CJD have a late age of onset (Mead et al. 2009). Therefore, the aged microglia model could be applied to the study of prion diseases. Initially conditioned medium from iron-fed microglia can be applied to neuronal cells and the expression and levels of prion protein could be measured by luciferase reporter assays and western blotting. The propensity and levels of aggregation of the prion protein in neuronal cells under the influence of iron-fed microglia could also be measured. If those are affected this could be pursued further by identifying which molecules present in the medium act to elicit those changes.

5.8.3 Multiple sclerosis

Microglial cells are thought to play a significant role in MS but it has not been established whether the role is mostly beneficial or detrimental (Luo et al. 2017). Iron-rich microglia have been detected at the edges of lesions in MS and are thought to drive inflammation (Gillen et al. 2018). Iron-fed microglia could be applied to other models of MS in order to simulate this effect. *In vitro* co-culture models would be especially suitable for this purpose. For example, in CNS spheroid cultures myelination can occur in a manner that is

very similar to *in vivo* and introducing microglia to this model can be useful in understanding the actions of this cell type in the context of MS models (Vereyken et al. 2009). Firstly, it could be observed whether iron-fed microglia have any effect on myelination through their conditioned medium. Secondly, demyelination can be experimentally induced in this model by using antibodies to myelin oligodendrocyte glycoprotein, IFNγ or lysophosphatidylcholine (Kipp et al. 2012). The speed of re-myelination in the presence of iron-fed microglia conditioned medium as compared to controls could be measured through electron microscopy of fixed sections of the spheroids. If a difference in the speed is detected, different components of the secretory profile of the iron-fed microglia could be tested to replicate the effect. The same experiments could also be attempted in the presence of iron-fed microglia in the spheroids in order to incorporate cross-talk between the cell types and to potentially assess if microglial phagocytosis can also play a role in de/remyelination.

5.8.4 Huntington's disease

Microglia in HD have been found to be activated in regions affected by the disease with an increase in microglial density correlating with an increase in neuronal loss (Yang et al. 2017). The cause of this activation are thought to be mutant huntingtin aggregates in neurons but the result has largely not been agreed upon – they could fulfil not only a detrimental but also a protective role (Crotti and Glass 2015; Yang et al. 2017). However, expression of mutant huntingtin in microglia also seems to alter their behaviour, as it leads to increased levels of activation without any exposure to neuronal aggregates (Yang et al. 2017). Interestingly, similar to aging, dystrophic microglia in HD have been found to accumulate ferritin (Simmons et al. 2007). It is possible that this occurs because of huntingtin's putative role as a regulator of the levels of iron in the cell as a type of iron response protein and its' mutation leading to dysregulation and the subsequent accumulation (Hilditch-Maguire et al. 2000; Lumsden et al. 2007). As it has been previously discussed, accumulation of iron in microglia can trigger a more activated state without any exogenous stimulation. Therefore iron-fed microglia could be used in the study of HD in order to unravel the role of these cells in the pathology of the disease. The conditioned medium from iron-fed microglia could be applied to neurons expressing mutant huntingtin and their viability assessed. If that is affected, the possible involvement of microglial secreted factors in the assembly of toxic species of huntingtin and subsequent impairment of the proteostasis of neurons could be assessed by measuring oligomer levels and ER stress as described by (Leitman et al. 2013).

5.9 Conclusion

The aged microglia model presented in this thesis is novel and investigates iron accumulation – a common but generally un-investigated phenomenon in aging. They were found to replicate aging microglia in many ways and importantly, to replicate microglial behaviour in neurodegenerative disease.

Even though the studies presented in this thesis show that iron-fed microglia are applicable to the study of neurodegeneration it is important to understand that the model is still in its infancy. A lot of future work is needed to refine the model, most importantly optimising the method for generating the iron-fed phenotype for different cell lines and microglia from other species. Furthermore, it is essential to measure changes in other senescence factors and to perform RNAseq on the different iron-fed microglia models. That would allow for the model to be optimised for different applications in the study of aging and neurodegenerative disease.

Aged microglia have been implicated in many neurodegenerative conditions and the versatility of this model means that aged microglia have great potential to be incorporated into both *in vitro* and *in vivo* studies of these diseases and also aging itself. One of the biggest potential impacts this model could have is in pioneering reversal of the effects of aging in microglia. Successfully reversing the iron-fed phenotype could lead to not only greater understanding of the aging process but also in powerful brain anti-aging treatments that could protect older individuals from developing debilitating neurodegenerative diseases.

6. Bibliography

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7. Appendix

7.1 Iron, Aging, and Neurodegeneration

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Review

Iron, Aging, and Neurodegeneration

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Abstract: Iron is a trace element of considerable interest to both chemistry and biology. In a biological context its chemistry is vital to the roles it performs. However, that same chemistry can contribute to a more deleterious role in a variety of diseases. The brain is a very sensitive organ due to the irreplaceable nature of neurons. In this regard regulation of brain iron chemistry is essential to maintaining neuronal viability. During the course of normal aging, the brain changes the way it deals with iron and this can contribute to its susceptibility to disease. Additionally, many of the known neurodegenerative diseases have been shown to be influenced by changes in brain iron. This review examines the role of iron in the brain and neurodegenerative diseases and the potential role of changes in brain iron caused by aging.

Keywords: synuclein; amyloid; prion; Alzheimer's disease; Parkinson's disease; transmissible spongiform encephalopathy; ferrireductase; microglia

1. Introduction

While the atomic nature of matter might be an absolute given in the 21st century, the role of metal atoms in biological systems remains a developing field. In particular, the difficulty of translating between chemical and biological systems remains central to advancing concepts that could actually lead to a better understanding of how our minds work. At the fundamental level, we also still need to understand how the movement of single electrons can have a significant impact on cellular mechanisms that

influence the way we age. In biological systems the movement of electrons is often dependent on metal ions and their role in enzyme activities or more fundamentally as co-factors in catalysis of various reactions. The regulation of such reactions can be both positive and negative as one results in maintaining cellular activity essential for life while the opposite can result in the generation of harmful reactive chemical species of oxygen or nitrogen. The production of reactive oxygen species (ROS) or nitrogen species (RNS) has been linked to changes in the brain associated with normal aging and also to diseases that can occur during aging such as the neurodegenerative diseases [1,2]. For many neurodegenerative diseases, especially the most common ones like Alzheimer's disease and Parkinson's disease, aging is a prerequisite for developing them [3,4]. While we can measure changes in various chemicals or reactions in the brain, the actual mechanism by which growing older makes us more susceptible to neurodegenerative diseases is a mystery. Yet, it has quite clearly emerged that changes in certain metals, especially iron, might be very important to both how the brain ages and to neurodegeneration.

Iron is the second most abundant metal on earth and has multiple oxidation states [5]. This makes it a leading candidate as a co-factor for enzyme-catalyzed reactions that require electron transfer. Iron can exist as Fe^{2+} (highly water soluble) or Fe^{3+} (much less soluble) in biological systems. Higher oxidation states are generated by some reactions but are much less stable than Fe^{2+} and Fe^{3+}. The redox potential of Fe^{2+}/Fe^{3+} is highly variable in biological systems and depends largely on the way it is coordinated within the structure of the ligands it binds [6]. This means that its utility is very high. Iron binding proteins can have different iron centers with specific classes of co-ordination [7]. The main classes are heme proteins, iron-sulfur proteins, and a class that is neither. This latter group includes iron storage and iron transport proteins, proteins coordinating a single Fe atom to histidine, glutamate or aspartate, or two Fe atoms in an oxygen-bridged center. The latter family includes many oxygen-binding proteins as well as Fe-oxidizing proteins such as ferritins.

Iron enters the body through the diet and is taken up via two main mechanisms [8]. The first (major) source of iron is through the metabolism of heme, which is absorbed at the intestinal apical membrane where the iron is released by heme oxygenase. Iron released in this way can directly enter the cellular free iron pool, where it can either enter storage in ferritin, or be transported outside the intestinal cell. The second uptake mechanism is at the brush border of the duodenum where it is first reduced to Fe^{2+} by the reductase Dcytb and then transported into the enterocytes by divalent metal transporter 1 (DMT-1) [9]. Iron taken up by this route enters the same pathway as heme iron. Once iron reaches the blood it is largely transported by transferrin. The main uptake mechanism involves the interaction of transferrin with its receptor at the cell surface. Internalization is through clathrin-coated pits, which directs transferrin to endosomes. There, the transferrin undergoes a conformational change as a result of the low pH of the endosomes causing iron release. The iron is reduced and transported to the cytosol. Iron uptake can also occur through DMT-1, which also transports other divalent metals [10].

Within cells, iron is associated with a variety of proteins as a co-factor, but is also stored. The main storage protein is ferritin, but other storage proteins exist such as hemosiderin [11,12]. Additionally, cells possess a pool of free iron known as the labile iron pool [13]. This pool is considered transient but necessary as the source of iron for newly synthesized proteins that require iron as a co-factor. The need to tightly regulate this pool of Fe^{2+} is considerable given the potential of free iron to catalyze radical generating reactions such as the Haber-Weiss reaction [14]. The production of radicals in the form of ROS and RSN has the potential to result in unwanted protein modifications and lipid peroxidation. These

modifications of proteins can also lead to misfolding of the proteins. Aggregation of proteins in the brain can potentially give rise to neurodegenerative diseases. The necessity of iron for many biological activities and the potential of iron to cause damage to cells must obviously be balanced. These processes deteriorate as we age. The consequences for the brain are particularly severe in this regard. However, the exact role of iron in either aging or neurodegeneration is still under investigation and remains uncertain. The evidence for its role is explored in this review.

2. Iron in the Brain

The brain contains significant amounts of iron. However, the distribution is not uniform. Regions such as the substantia nigra and the globus pallidus have the highest levels, exceeding that of the liver, the main site of iron storage in the body [15–17]. According to cell type, oligodendrocytes have the highest iron content and astrocytes have very low cellular iron [18]. The main storage protein for iron is ferritin, which is composed of a mixture of H- or L-ferritin monomers (heavy or light chain), and varies between cell types as to which is expressed predominantly. Neurons express predominantly H-ferritin while microglia express L-ferritin [19,20]. Some neurons, such as those of the substantia nigra, express neuromelanin. Neuromelanin is synthesized from L-Dopa in dopaminergic cells and forms stable complexes with Fe(III). Thus cells expressing neuromelanin are likely to have increased iron storage [21,22].

Iron storage increases with age in the brain [23]. Again, this is not uniform and some regions show greater increases than others. Studies of ferritin levels have indicated high increases in the cortex, globus pallidus, and substantia nigra. In other regions such as the locus coeruleus the iron concentrations remain low throughout life [24]. The main cell types accumulating iron with age are microglia and astrocytes [23]. These changes are found in many brain regions including the cortex, cerebellum, hippocampus, and basal ganglia. In contrast, there is little change in iron in oligodendrocytes despite their higher concentrations of cellular iron. The origin of increased brain iron remains unclear. However, increased vascularization of the brain may increase the chance of iron exchange between the blood and tissues of the brain, thus increasing the concentration of iron [25,26]. Whatever the reason, an increase in a potentially oxidative metal in the brain increases the chances of deleterious reactions. It is probably no coincidence that the regions associated with changes in iron are also those associated with several neurodegenerative diseases.

3. Neurodegenerative Diseases Linked to Iron

This review is aimed at understanding the potential role of iron in aging and neurodegeneration. However, despite a potential involvement in more common diseases, there are instances of quite specific diseases directly linked to iron and its metabolism. Some of these diseases have successful treatments that involve the use of iron chelators.

Friedreich's Ataxia is the most common of the ataxias and affects adolescents [27]. The disease is caused by mutations in the gene for frataxin, a protein found in the mitochondria that is associated with the assembly of Fe-S clusters and may act as an iron chaperone [28]. Mitochondria are also considered to be the main site of iron accumulation in Friedreich's Ataxia [29]. Recent research has suggested that frataxin acts as an allosteric mediator of Fe-S cluster assembly [30]. It is found in tissue with high

metabolism such as the heart and dorsal root ganglia parallel to the spinal cord [31]. The mutation in the gene involves trinucleotide repeat insertions (GAA) in the first intron. Severity of the disease appears to be linked to the number of insertions, which decrease the expression level of frataxin [28]. Patients have severe neurological problems that seem to be linked to problems with excess iron. Treatment of patients with the iron chelator deferiprone caused marked reduction in iron levels within the dentate nuclei and alleviated symptoms including manipulative dexterity, speech fluency, reduction in neuropathy, and ataxia gait [32].

Neuroferritinopathy is a rare, dominantly inherited disease associated with mutations in the gene for ferritin [33]. The disease has early and late onset forms and is associated with motor symptoms, spasticity and cognitive deficits [34]. The cause is usually a frameshift mutation in exon 4 for the L-ferritin gene, which causes a conformational change in the *C*-terminus of the molecule and alters its ability to store iron [35,36]. The mutation results in iron/ferritin-rich aggregates forming in cells [37]. Mouse transgenic models show a strong relation between functional changes and abnormal iron metabolism [38]. However, attempts to treat the disease with chelation therapy have so far been unsuccessful [39].

Many of the iron-storage diseases are inherited and pantothenate kinase-associated neurodegeneration (PKAN, formerly known as Hallervorden-Spatz syndrome) is another such disease [40]. It is characterized by Parkinson's-like symptoms as well as significant mental abnormalities. It develops in childhood and is usually fatal. It is associated with mutations in the gene encoding pantothenate kinase 2 (PANK2) [41]. For this reason the name was changed from Hallervorden-Spatz syndrome. PKAN, now the more common term for the disease, is considered one of a family of diseases called NBIA (neurodegeneration with brain iron accumulation) [42,43]. PKAN brains show specific areas with high levels of iron accumulation, including the globus pallidus and the substantia nigra [44]. The exact relation of iron accumulation to disease progress is unclear. It has been suggested that the disease results in coenzyme A deficiency and subsequently increased cysteine, which is then able to chelate the iron [45]. However, treatment of patients with iron chelation therapy caused reduced iron accumulation but no change in symptoms. Therefore, the role of iron in the disease is unclear and may just be a symptom rather than a cause [46].

NBIA diseases also include neuroferritinopathy, aceruloplasminemia, beta-propeller protein-associated neurodegeneration, and a number of inherited diseases [43,47,48]. The incidence of NBIA disease continues to increase and clearly shows the importance of understanding iron metabolism and its potential link to neuronal loss. Many NBIA diseases are childhood diseases and are clearly not linked to aging. However, they are illustrative of the potential impact that accumulation of iron in the brain due to natural aging processes has on the vulnerability of neurons to iron-associated cell death.

4. Iron in Alzheimer's Disease

Alzheimer's disease (AD) is both the most common dementia and also the most common neurodegenerative disease. The greatest risk factor for the disease remains aging. The risk of developing the disease accelerates as we grow older, with those over 85 having almost a 1 in 2 change of developing it. AD is progressive and irreversible and results in memory loss, cognitive decline, a variety of psychological changes including anxiety, depression, and aggression, and eventual loss of physiological

functions due to dementia [49]. Several brain areas are affected including the hippocampus, temporal lobe, and frontal cortex. Particularly, the cholinergic innervation is severely disrupted [50]. The exact cause of the disease remains contentious. There are inherited and sporadic forms of the disease. The inherited forms, although much rarer, point towards the proteins most likely to be at the heart of the disease [51]. The lead hypothesis of the cause of AD is the amyloid cascade hypothesis [52]. The formation of aggregated forms of β-amyloid (Aβ) is a common hallmark of the disease. Aβ is formed from a large precursor called APP (amyloid precursor protein). Inherited mutations linked to AD are found in the APP gene or in the genes of proteins associated with processing APP to form Aβ. The formation of Aβ aggregates in the brain is likely to play a significant role in the disease and may be associated with the neuronal loss observed [52]. The other significant protein in AD is tau, a microtubule-associated protein [53]. In AD tau becomes hyper-phosphorylated and forms paired-helical filaments (PHF), also known as tangles. There are suggestions that PHF are the true cause of AD. However, it could also be that some interplay between Aβ and tau may be important [54].

APP is a transmembrane protein and as the parent protein for Aβ there is significant debate over its role in the cell and whether altering its metabolism could be the significant causal effect that initiates AD [55]. A significant consideration in this debate is that APP binds metals [56]. APP contains a classical type II copper-binding domain in its *N*-terminus. The residues His147, His151, and Tyr168 were identified as the copper coordination sphere of the binding region within the E1 region of the protein [57]. The His-X-His motif is similar to that seen in Cu/Zn superoxide dismutase. APP has the potential to reduce Cu^{2+} to Cu^{+}. The E2 domain of APP has also been suggested to have two metal binding sites [58]. Of these, Zn^{2+} is thought to be coordinated between His382, His432, and His436. Copper can also bind this site with the addition of His313 to the three His of the Zn site. A second, low-affinity site is coordinated by Glu387, Asp429, and His458. There has also been a report that the E2 site interacts with iron. A suggested site for iron binding around Glu337 and Glu340 is still debated [59]. The idea that APP could function as a ferroxidase [60] has been dismissed in favor of the suggestion that APP can regulate iron export from the cell (Figure 1) [59,61].

As well as physical interactions between metals and APP, there has been considerable research on the cellular implications of those possible interactions. Of considerable interest are those that might alter or initiate the disease (Figure 2).

Interaction of APP with copper is thought to alter both its dimerization and the rate of cleavage to form Aβ [62,63]. However, the aggregation of Aβ is also thought to be influenced by its interaction with copper and zinc [64–66]. Cu, in particular, is considered to accelerate aggregation. This led the research group of Ashley Bush to investigate whether copper chelators such as clioquinol could inhibit Aβ aggregate accumulation and possibly be a treatment for AD itself. While clioquinol proved effective in depletion of Aβ in transgenic mice [67], it unfortunately did not progress in clinical trials [68].

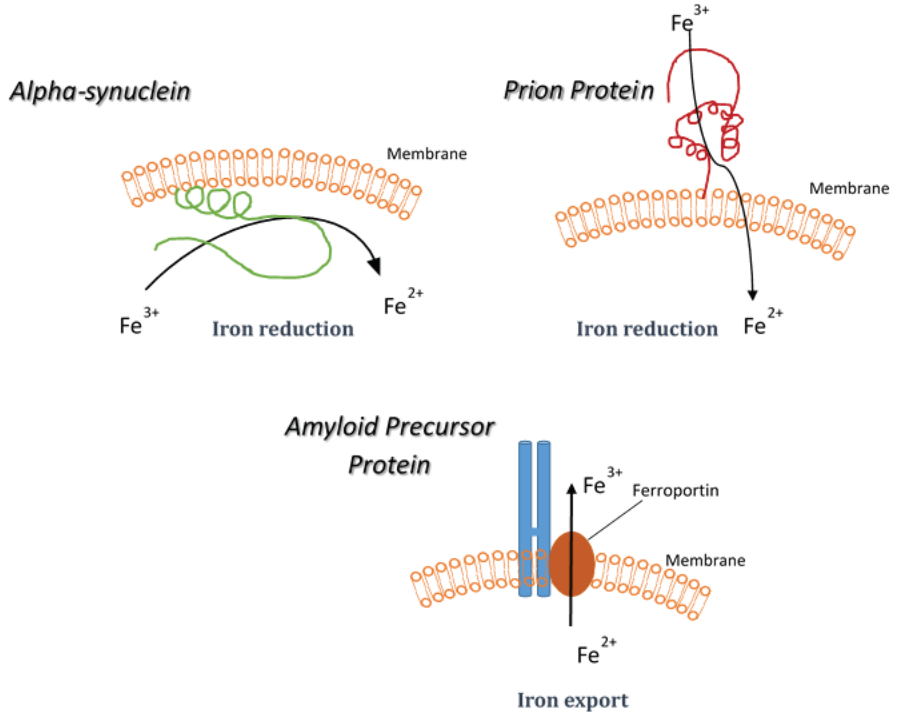

Figure 1. Several of the key proteins associated with neurodegeneration have been suggested to have roles associated with iron. This figure summaries these suggestions. α-synuclein is associated with PD and has been shown to bind iron and reduce it, thus converting Fe^{3+} to Fe^{2+} which is utilized for many cellular activities. α-synuclein, like most ferrireductases, is associated with the inner face of the cell membrane. There have also been reports that the prion protein, associated with diseases like CJD, can also act to reduce iron as a ferrireductase. Lastly, the amyloid precursor protein (APP) the precursor to beta-amyloid known from AD, has been shown to enhance iron export out of cells. This process requires the presence of ferroportin, which mediates the iron export process.

While the relation of copper and zinc to AD and the APP protein has proven quite solid, evidence supporting a link between iron and AD has taken much longer to emerge [69]. Elevated brain iron has been reported in patients with AD [70,71]. However, as is clear from this review, this is not unique to AD. Iron accumulation in AD occurs without a parallel increase in ferritin [72]. IRP proteins normally respond to increased levels of iron by interacting with genes containing the IRE element and causing regulation of a number of proteins in a coordinated fashion. This usually ensures that the transferrin receptor and ferritin are regulated together. In AD it appears there is a dysfunction of this system that leads to increased free iron that would otherwise be stored in proteins such as ferritin [73]. In a recent clinical study of cerebral spinal fluid (CSF) from AD patients, it was found that ferritin levels were predictive of cognitive status [74]. While not being distinctly elevated in all cases of AD, declining cognitive status from mild cognitive impairment (MCI) to AD was associated with altered ferritin levels and was also greater with younger onset of AD. This would suggest a relationship between high iron burden in the brain and an early age for AD onset. Additionally, it was found that ferritin levels were higher in individuals with the APOE protein allele ε4 [74]. This allele is an epigenetic marker for greater risk of AD. However, the link between APOE and iron metabolism cannot currently be explained.

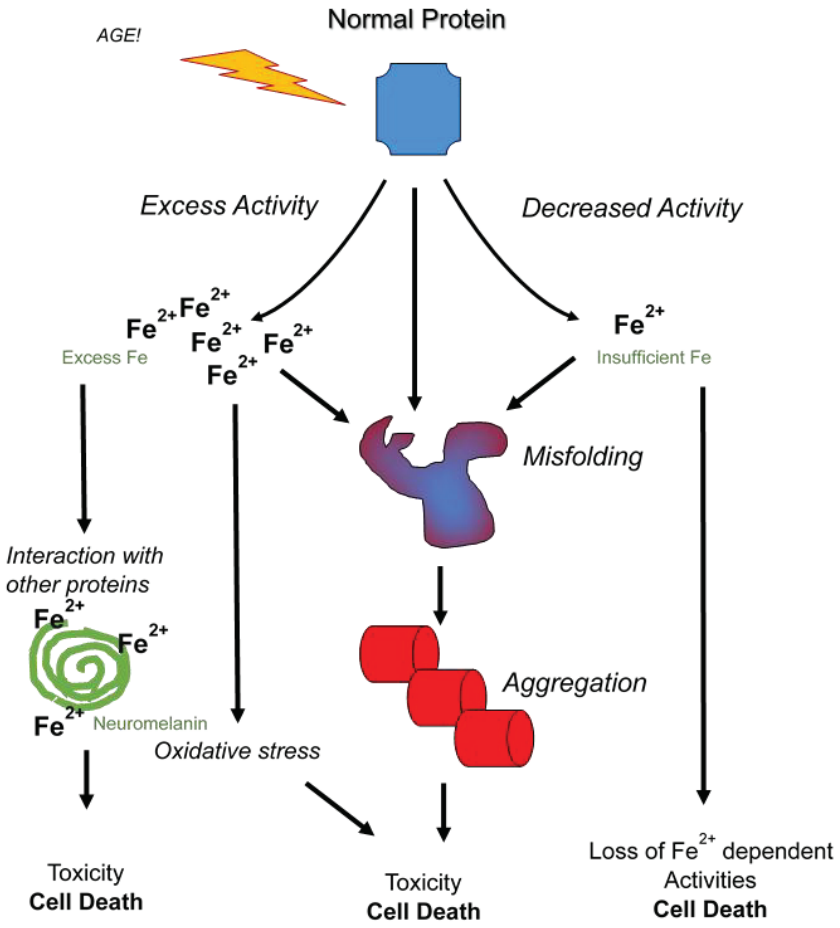

Figure 2. While numerous proteins associated with neurodegeneration may have a role in iron metabolism in the healthy cell, under certain conditions these processes might be disturbed. This diagram represents a summary of the potential changes that can occur during aging which might disturb the way iron is either utilized or its interactions with other proteins. Changes in the protein might result in changes in levels of iron, particularly Fe^{2+}. The protein may become overexpressed, resulting in the formation of excess Fe^{2+}, or some kind of damage to the protein might result in lost activity and reduced Fe^{2+}. Additionally, aging effects might result in the misfolding of the protein, either as a result of an increase or a loss of interaction with iron or simply through another process. Misfolded protein could then aggregate and cause toxic damage to cells. In the case of excess generation of Fe^{2+}, there could be abnormal interaction with other proteins such as neuromelanin or the Fe^{2+} could result in oxidative stress. In both cases the end result could be the generation of toxic species that lead to cell death. Lastly, in some cases the lack of Fe^{2+} might result in a breakdown of essential cellular process necessary for cell survival.

As mentioned above, Aβ binds metals. While there has been a focus on copper, there is also evidence for an interaction with iron [72]. While this could mediate toxicity or oxidation events, there has also been a suggestion that the interaction could be protective. In this case the Aβ would sequester the iron and prevent it mediating toxic damage. While this may seem paradoxical given the "bad" reputation of Aβ, it is actually true that free iron is highly toxic and Aβ can accumulate without any apparent cell

loss [75,76]. This observation, although suggesting a positive role for Aβ in AD, does not then explain how iron might be toxic.

Mitochondria are the organelles most likely to show damage in AD [77]. As the site of heme synthesis in the cell, mitochondria are a major site for iron handling. Mitochondria are the major source of oxygen radical generation in cells. Damage to mitochondrial-specific proteins, which can increase free iron, has been shown [78]. Studies of mitochondrial DNA suggest that there is a greater turnover of the organelle in AD [79]. This supports the fact that there is mitochondrial abnormality in AD [80]. There is no direct evidence that this dysfunction is causal rather than a result of the damage that leads to cell death in AD or that there is a direct link between mitochondria and iron. Further research may bring into focus the causal links between these different factors. However, it is clear that iron disturbance is one important part of trying to decipher the mystery behind AD.

5. Synucleinopathies and Iron Reduction

Synucleinopathies are a family of diseases associated with the deposition of the protein α-synuclein in an aggregated form in CNS tissue [81]. These diseases include Parkinson's disease (PD), Multiple System Atrophy, and Dementia with Lewy Bodies (DLB) [82,83]. Parkinson's is very well known, being the most common of the non-dementing neurodegenerative diseases. It is associated with loss of dopaminergic neurons from the substantia nigra, which results in a movement disorder [84]. PD has a long association with disturbances to iron metabolism [85]. However, any mechanistic relation between the disease and iron metabolism has yet to be firmly established. PD, like AD has sporadic and inherited forms. The inherited forms are related to mutations in the genes of a long list of proteins which include α-synuclein, parkin, leucine-rich repeat kinase (LRRK), PINK-1, and DJ-1 [86]. The inherited forms can be either early or late onset (depending on the mutation). The clinical symptoms of PD include resting tremors, muscle rigidity, and bradykinesia [87]. Treatments are generally focused on restoring some amount of dopamine to the patient as the loss of dopaminergic neurons is the principal change that results in the majority of symptoms. These treatments range from supplying the dopamine precursor (L-Dopa) to altering dopamine transport and breakdown [88].

Changes in PD patient brains include increased levels of Fe(III) and reduced levels of the Fe(III)-binding protein ferritin [89]. Increased iron deposits in the substantia nigra are associated with α-synuclein-positive Lewy bodies in PD and neurodegeneration with brain iron accumulation [90,91]. However, there are also changes in other metals in PD such as zinc in the substantia nigra and high levels of copper in the cerebrospinal fluid [92]. There is also evidence from epidemiological studies that increased incidence of PD is associated with environmental metal exposure. Individuals with chronic industrial exposure to copper, manganese, or iron have an increased rate of PD [93]. In experimental models, alterations in metal homeostasis were observed with toxin-induced animal models of PD. An accumulation of iron was observed in the substantia nigra of MPTP-treated mice, which is likely a result of the observed upregulation of transferrin receptor expression and iron uptake [94,95]. Although these observations may be consequences of the disease progression, experimental studies using $FeCl_3$ injected directly into the substantia nigra of rats resulted in a 95% reduction in striatal dopamine and altered behavior, supporting the idea that iron initiates dopaminergic degeneration in PD [96].

While observation of changes in metal levels in a disease might be interesting, without a mechanistic link the findings remain at best a marker of change. The increased cellular iron is likely to come about through changes either as a result of intake or release. However, with regards to the death of dopaminergic neurons, there is also the possibility that it is not the absolute amounts that are important but the ratio of Fe(II) to Fe(III). Similarly, alteration in the interactions of iron with various proteins may also be critical. For example, the substantia nigra is characterized by high levels of neuromelanin, the dark pigmented protein that gives the substantia nigra its name. Studies of PD patients have shown high levels of iron associated with neuromelanin granules [97]. This could suggest that neuromelanin traps redox-active Fe^{2+}, which is then able to initiate the oxidative process [98,99]. However, another study suggests that the iron leaves the neuromelanin and migrates to the cytosol in PD [100].

Cellular responses to fluctuating iron levels are regulated by iron regulatory proteins (IRPs) that bind to iron response elements (IRE) in RNA [101]. However, they also respond to increased levels of oxidative stress and can then alter cellular protein expression to cause an increase in the free iron pool [102]. The implication is that oxidative stress can cause a dysregulation of iron metabolism. The read-out for such a change would be increased iron levels without a corresponding change in iron-binding proteins such as ferritin. This has been observed in PD [103]. As well as potential interactions of iron with other proteins, iron could also alter dopamine specifically, causing the generation of toxic dopamine byproducts that then kill dopaminergic cells specifically. One of the metabolic products of dopamine 3,4-dihydroxyphenylacetaldehyde (DOPAL) can induce aggregation of α-synuclein in the presence of iron [104]. DOPAL can also generate reactive oxygen species in the presence of iron [105].

Synucleins have been linked to metals. Firstly, all three synucleins bind copper [106,107]. α-synuclein also binds other metals including iron [108,109]. Exposure of α-synuclein to metals during extensive shaking can also accelerate its aggregation [110]. This creates a dichotomy with regards to α-synuclein in terms of both its potential normal cellular activity and its aggregation, which is associated with pathological states such as in PD. α-synuclein toxicity is mostly associated with copper [111,112]. During the aggregation process, copper induces the formation of a unique stellate oligomer that is highly toxic to neuronal cells in culture [111]. Exposure to iron does not have this effect, although some studies have shown that iron can accelerate aggregation of α-synuclein [113]. The aggregation of α-synuclein in cells is associated with the increased expression levels of the protein. However, increased expression of α-synuclein has also been shown to increase cellular iron concentrations [114].

Recent studies have shown that α-synuclein can bind both copper and iron simultaneously [109]. When α-synuclein binds copper it is able to undergo redox cycling, as shown by cyclic voltammetry [106]. The implication is that α-synuclein can use copper to move electrons. Further studies have shown that α-synuclein can cause the reduction of iron (Figure 1) [109]. Kinetic analysis with purified recombinant protein has shown that this activity is enzymatic, potentially making α-synuclein a ferrireductase. The implication is that the potential of α-synuclein to generate Fe^{2+} in cells may be its normal cellular role. What this means for the pathology of the synucleinopathies currently remains unclear. However, there are two distinct possibilities. First, increased expression of α-synuclein could result in excess production of Fe^{2+}, which could then initiate catastrophic oxidative processes in the cell, interact with other proteins such as neuromelanin, or simply be neurotoxic (Figure 2). The

alternative is that when α-synuclein is highly expressed in cells and begins to aggregate the protein becomes functionally inactive but may still bind Fe^{3+}, thus sequestering iron that is needed for normal cellular activities such as the synthesis of dopamine. Give the potential role of Fe^{2+} in numerous aberrant processes, the former possibility is more likely. However, as with the general role of iron in PD, the role of iron reduction in PD is still unclear and further research is needed to understand what causative role there is, if any.

6. Prion Diseases

Also known as transmissible spongriform encephalopathies, prion diseases are rare [115]. They are more widely known because of the concurrent outbreaks of both bovine spongiform encephalopathy (BSE) in cattle and variant Creutzfeldt-Jakob disease (vCJD) in humans [116,117]. The potential transmissibility of prion diseases remains an ever-present concern. However, the major form of human prion disease (sporadic form of Creutzfeldt-Jakob disease) is still very rare and is not naturally transmissible [118]. Prion diseases can either be inherited (through mutations in the *Prnp* gene), sporadic (with no known cause) or transmissible. The transmissibility has largely been demonstrated experimentally, but may be a result of misadventure such as with vCJD or Kuru (a disease spread by ritual cannibalism). The disease transmissibility is associated with an abnormal isoform of the prion protein [119]. The prion protein (PrP^c) is a cellular copper-binding glycoprotein expressed at the cell surface [120,121]. The function of PrP^c remains controversial, but the strongest evidence supports its role as an antioxidant protein associated with increased cell viability [122–124]. The protease-resistant isoform of PrP^c often accumulates at high levels in the central nervous tissue of patients and animals with prion diseases. Therefore, this abnormal isoform (PrP^{Sc}) is a hallmark of the disease. It is tightly associated with the infectious agent and may be a direct cause of the neurodegeneration seen in the disease [125].

Following the discovery of the copper binding capability of PrP^c, extensive studies were carried out to assess the relation of PrP^c and prion diseases to metal homeostasis. The strongest associations were found between prion diseases and copper metabolism but also manganese metabolism [126]. The latter was somewhat unexpected and remains controversial. While copper has been associated with normal PrP^c activities, the data suggest that manganese binds to PrP^c in disease and can cause its conformational change [127,128]. Manganese binding to PrP also increases its survival in the environment and increases its ability to cause prion infection in cells [129].

Studies looking at the levels of trace elements in the brains of animals with BSE, sheep scrapie, patients with CJD or vCJD, and rodents experimentally infected with scrapie all show a similar trend [130–132]. They indicate reduced levels of copper and increased levels of manganese. Similar changes have also been observed in the blood. A recent study has shown increased manganese in prion plaques in experimental hamsters [133]. Other trace elements were also studied and very little difference was observed for any other metal. These studies included Fe, which showed no changes in any of the tissues or diseases analyzed.

Despite these studies showing no changes in iron, there are others that suggest a strong role for PrP in iron metabolism [134]. An initial study suggested increases in both ferritin and iron response proteins in astrocytes in rodent scrapie models [135]. The research group of Nina Singh has extensively studied the links between Fe and prion disease. Their findings initially suggested that PrP could influence iron

uptake [136]. Cells overexpressing PrP showed increased levels of intracellular iron. Further studies suggested that CJD patients have increased levels of iron, while PrP knockout mice have decreased iron levels [137,138]. A number of studies have shown that the divalent metal transporter-1 (DMT-1) is altered in both PrP-knockout and prion disease [139]. This protein is linked to the transport of multiple divalent metals and not just iron. However, changes in this protein could explain changes in intracellular iron. Despite the changes in uptake and storage of iron suggested by these studies, it has also been suggested that PrP^c itself is a ferrireductase (Figure 1) [140]. The implication is that PrP^c causes conversion of Fe^{3+} to Fe^{2+}, which is then more likely to be transported into the cell, thus resulting in increased cellular iron. However, unlike alpha-synuclein, for which kinetic studies on purified protein suggested iron reduction is a result of true enzymatic catalysis109], studies on the ferrireductase activity of PrP^c have only been performed on cell extracts [140]. As PrP^c has already been suggested to be associated with redox balance in cells [123], this observation may only be reporting a secondary effect rather than a direct function of PrP^c in iron reduction.

Unlike other diseases where the association with iron metabolism has been robustly established, the link between iron and prion diseases remains unclear. The majority of the data do not support this link. Iron binding to PrP is weak and highly pH dependent, implying there is not likely to be a physiological interaction of PrP^c and iron. However, it will be interesting to see if further data confirm a role of PrP^c in iron metabolism.

7. Microglia and Aging

While iron metabolism is of interest to specific diseases, general aging can cause other changes upstream that also impact on the same diseases. Here again, changes in the way iron is handled by the brain impact on the cellular environment. An example of the changing environment in the aging brain is a change in the supporting cells in the brain, including microglia [141]. Microglia are the resident macrophages of the brain and are its first and main form of active immune defense. Healthy microglia are very sensitive to their environment, constantly surveying for and phagocytosing any foreign material or cellular debris that they encounter. Additionally, they are capable of releasing cytotoxic substances that can kill neurons that are damaged or infected. Such substances include H_2O_2, nitric oxide, inflammatory cytokines, proteases, and neurotransmitters. Activated microglia can act as antigen-presenting cells and activate T-cells. After an infection has been dealt with, microglia can recruit cells involved in neuronal repair and secrete anti-inflammatory cytokines [142]. The idea of aging microglia stems from histological observations of healthy aged brains where the cells often develop dystrophic phenotypic characteristics [143]. Resting microglia have a ramified morphology with many fine processes extending from the cell body. Dystrophic microglia found in aging brains lose this fine process of ramification (Figure 3). Dystrophic microglia often develop abnormally shaped processes with spheroidal swellings and cytoplasmic fragmentation (cytorrhexis) [143]. Dystrophic microglia have also been associated with increased release of toxic ROS and inflammatory cytokines and impaired phagocytic ability [144,145]. Cytokines are small proteins involved in intercellular signaling. They are released by a multitude of cell types including immune cells like microglia and bind to cell surface receptors on other cells where, through signaling cascades, they alter the transcriptional profile of the target cells. The proinflammatory cytokines found to be released by dystrophic microglia include IL-6, TNF-α, and IL-1β [145].

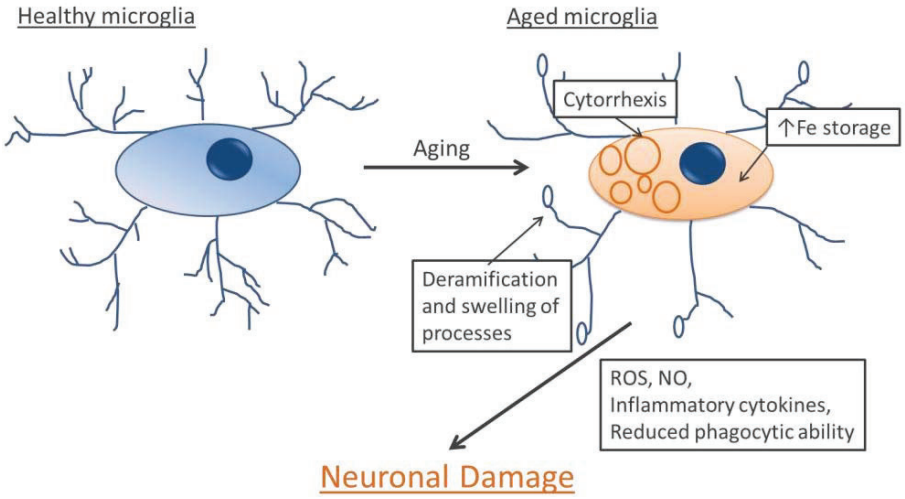

Figure 3. Age-related changes in microglia. When microglia age they lose some of their processes and develop abnormalities in others. Additionally, they often exhibit cytoplasmic fragmentation. They also store more iron. Their increased release of neurotoxic substances and reduced ability to phagocytose debris and toxic protein aggregates leaves neurons vulnerable.

Ferritin, the main iron storage protein in the brain, is highly expressed in microglia [19]. Microglia are the main cells in the brain that store iron. One way to identify dystrophic microglia, apart from their morphology, is their higher levels of iron storage, demonstrated by the expression of the iron storage protein ferritin. Additionally, an outward rectifier K^+ channel called Kv1.3 has been found to increase expression in dystrophic microglia in aged mouse brains [146]. Dystrophic microglia become more prevalent with human aging and have been found to increase in a variety of diseases including AD [147] and Huntington's disease [148]. There is also evidence that the chronic inflammation that accompanies neurodegeneration leads to local increases in microglia with high iron and ferritin content, possibly due to iron scavenging [149]. The association between increased iron storage and an altered microglia phenotype, particularly a dystrophic one, suggests a possible causative role for iron. As iron can damage cells, dystrophic microglia possibly develop as a direct result of increased iron storage.

8. Microglia and Neurodegenerative Diseases

The presence of healthy glial cells is critically important to neuronal wellbeing. Microglia maintain homeostasis in the healthy brain and fight infection when it is present through a complicated system of signaling molecules. The importance of microglia to neurons is supported by a higher incidence of dystrophic microglia and microglial apoptosis in AD [150]. The inflammation of the nervous system in neurodegenerative disease was thought to be due to activated microglia. However, dystrophic microglia also have impaired neuroprotective ability and generate the low but sustained release of inflammatory factors seen during neurodegeneration.

The effect of dystrophic microglia on the pathogenic changes occurring in Alzheimer's disease is not well understood. Over the progression of the disease, microglia seem to change from exerting a neuroprotective function to being closer to a classically activated state. This change in phenotype may

result in microglial neurotoxicity or alternatively in dysfunction that prevents the cells from fulfilling their protective role [150]. Dystrophic microglia have been co-localized with neurofibrillary tangles in AD brains [151]. Microglia with impaired phagocytic and motility functions have been co-localized with Aβ deposits in mouse models of Alzheimer's disease [152]. Healthy microglia have been shown to take up Aβ and also to release enzymes that degrade it [153]. However, dystrophic microglia have impaired Aβ phagocytic ability [144]. If healthy microglia are activated with LPS, they help reduce the Aβ burden in the brains of mice [154]. Pathologically activated microglia release pro-inflammatory cytokines and reactive oxygen species that can make neurons more sensitive to Aβ toxicity [155]. Dystrophic microglia have been found to be hyper-responsive to stimulation [145]. Inflammatory cytokines have been shown to increase the expression of APP in neurons, which can result in increased production of Aβ through also favoring the amyloidogenic APP processing pathway [156–158]. The microglial p40 subunit of IL-12 and IL-23 has been found to be elevated in AD brains and to correlate with a worse Aβ pathology [159].

9. Conclusions

Iron is a two-edged sword for biological systems—essential for many cellular activities, but also able to cause damage to macromolecules or disrupt sensitive processes. In the brain this balance is even more delicate given the irreplaceable nature of neurons. Research into the role of iron in both disease and normal activities in the brain will continue. However, the changes and impact of iron during aging and within neurodegenerative diseases is now well established.

Author Contributions

DMA and DRB wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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